The role of the *Streptococcus pneumoniae* capsule in interactions with complement and phagocytes

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DECLARATION

I, Catherine Jane Hyams confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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ABSTRACT

The *Streptococcus pneumoniae* capsule is an essential virulence factor and it is ideally situated to modulate interactions between the bacteria and host immune cells. Using isogenic unencapsulated mutants, flow cytometry assays and a mouse septicaemia model, this thesis has assessed the effects of the capsule on the interactions of *S. pneumoniae* serotype 2 and 4 strains with complement factors and phagocytes. Overall, these data demonstrate that the capsule inhibits complement activity but this only partially contributes to the effects of the capsule on neutrophil phagocytosis and virulence during septicaemia. Furthermore, interactions with macrophages were also found to be complement-dependent and independent, resulting in differences in both phagocytosis and inflammatory responses both *in vitro* cell line and *in vivo*.

I also investigated whether capsular serotype affects *S. pneumoniae* interactions with the host immune response using otherwise isogenic TIGR4 strains expressing capsular serotypes 4, 6A, 7F, 23F. These data demonstrate that resistance to complement mediated immunity is associated with capsular serotype, and hence this might be one mechanism by which capsular serotype could affect relative invasiveness of *S. pneumoniae* strains. Non-capsular genetic background was also found to affect complement mediated immunity, and importantly, relatively invasive strains were on average more resistant to complement than weakly invasive strains.

Overall the results in this thesis demonstrate that the *S. pneumoniae* capsule aids evasion of both complement dependent and independent immune mechanisms, and that serotype-dependent differences in the effects on immunity could partially explain variations in virulence between strains.

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ABBRIEVIATIONS

AF	Alexa Fluor
AM	Alveolar macrophage
ANOVA	Analysis of variation
ATP	Adenosine triphosphate
BALF	Bronchoalveolar lavage fluid
Bf	Complement factor B
bp	Base pair
BSA	Bovine serum albumin
C1q	Complement component 1q
C3	Complement component 3
C4BP	C4 binding protein
C57BL/6	C57 black 6 mouse strain
C9	Complement component 9
CAP	Community acquired pneumonia
CbpA	Choline binding protein A
CD	Cluster of differentiation
CFU	Colony forming units
CI	Competitive index
CO_2	Carbon dioxide
CPS	Capsule polysaccharide
CR	Complement receptor
CRP	C-Reactive protein
CSF	Cerebrospinal fluid
Da	Dalton
DAPI	4',6-diamidino-2-phenylindole
DAF	Decay accelerating factor
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3 Grabbing Non-integrin
dd	Double distilled

DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked ImmunoSorbent Assay
EM	Electron microscopy
ERK	Extracellular signal-related kinases
FAM-SE	5 - Carboxyfluorescein, succinimidyl ester
FBS	Foetal bovine serum
Fc	Fragment, crystallizable
FcR	Fc receptor
FH	Complement factor H
FITC	Fluorescein isothiocyanate
FI	Fluorescent Index
Gal	Galactose
GBS	Group B Streptococcus
G-CSF	Granulocyte colony-stimulating factor
Glc	Glucose
HBSS	Hank's Balance Salt Solution
HT	Heat treated (Complement Inactivated) serum
HRP	Horseradish peroxidase
iC3b	Inactivated C3b
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IFNγ	Interferon γ
IκB	Inhibitor of kappa B
IKK	IκB kinase
IL	Interleukin
IN	Intranasal
iNOS	Inductible nitric oxide synthase
IP	Intraperitoneal
IPD	Invasive pneumococcal disease
IQR	Interquartile range

IRAK	IL1R-associated kinase
JNK	Jun kinase
L-Glut	L-Glutamine
LPS	Lipopolysaccharide
LytA	Autolysin
MAC	Membrane attack complex
MARCO	Macrophage receptor with collagenous structure
MAPK	Mitogen activated protein kinase
MAPKK	MAPK kinase
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MCP-1	Monocyte chemotactic protein 1
MEK	Mitogen activated protein kinase kinase
MHC	Major histocompatibility complex
MLST	Multilocus Sequence Typing
MKK	Mitogen activated protein kinase kinase
MOI	Multiplicity of Infection
MyD88	Myeloid differentiation primary-response protein 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NALP	NOD-like receptors with pyrin domains
NETs	Neutrophil extracellular traps
NFκB	Nuclear Factor kappa B
NK	Natural killer T cells
NOD	Nucleotide-binding oligomerization domain
OD	Optical density
OR	Odds ratio
ORF	Open reading frame
PAFr	Platelet activating factor receptor
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin

PFA	Paraformaldehyde
PGM	Phosphoglucomutase
PMN	Polymorphnuclear cells
pNPP	p-Nitrophenyl phosphate
PsaA	Pneumococcal surface adhesin A
PspA	Pneumococcal surface protein A
RANTES	Chemokine (C-C motif) ligand 5
RAW 264.7	Mouse leukaemic monocyte macrophage cell line
RelA	Nuclear factor of kappa light polypeptide gene enhancer in B cells 3 (p65)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute Medium
S. pneumoniae	*Streptococcus pneumoniae*
SAP	Serum Amyloid P
SAPK	Stress activated protein kinase
SIGN-R1	Specific intercellular adhesion molecule-grabbing nonintegrin receptor 1
SD	Standard deviation
SR-A	Scavenger receptor A
ST	Sequence Type
T_H	Helper T cell
T_C	Cytotoxic T cell
TIR	Toll/IL1 receptor
TIRAP	TIR-associated protein
TLR	Toll like receptor
THY	Todd Hewitt broth with 0.5% yeast extract
TNF	Tumour necrosis factor
TRAF6	TNF receptor-associated factor-6
U	Units
UDP	Uridine diphosphate

VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
WT	Wild-type

CHAPTER 1 INTRODUCTION

1.1 BACKGROUND

1.1.1 Microbiology

Streptococcus pneumoniae are pathogenic Gram-positive, lancet-shaped cocci which usually grow in pairs, but may form short chains or grow singly. Individual cells are 0.5-1.25μm in diameter. *S. pneumoniae* is alpha-haemolytic when grown on blood plates, is non-motile and like other streptococci does not produce catalase, fermenting glucose to lactic acid. However, unlike other streptococci, *S. pneumoniae* does not display an M protein, hydrolyzes inulin and has a characteristic cell wall composition in terms of both teichoic acid and peptidoglycan. *S. pneumoniae* grows best in 5% CO_2 and in all growth conditions an external catalase source must be provided to neutralize the large quantity of hydrogen peroxide produced by *S. pneumoniae*. *S. pneumoniae* is part of the oral streptococcal group which includes *S. cristatus*, *S. infantis*, *S. mitis*, *S. oralis* and *S. peroris* and is differentiated from other viridians group streptococci based on colony morphology, optochin and bile sensitivity, inulin fermentation and agglutination with anti-capsular specific antibody raised in rabbits. However recent observations of pneumococci producing atypical reactions to these standard tests including optochin resistance (Munoz et al. 1990; Fenoll et al. 1994) and bile insolubility (Obregon et al. 2002) with the discovery of optochin-sensitive and bile soluble oral streptococci (Whatmore et al. 2000; Martin-Galiano et al. 2003), has presented difficulties with conventional identification methods. Alternative approaches to identification include the use of the *pia* locus, which encodes an iron uptake transporter, and is located in a pathogenicity island (PPI-1) (Brown et al. 2001). However it

is likely that a combination of 2 or 3 unique loci would be needed to confirm diagnosis of *S. pneumoniae*. Recently it was suggested that *S. pneumoniae, S. mitis* and *S. pseudopneumoniae* evolved from a common ancestor by a process of genome reduction, providing an explanation as to the difficulties encountered in differentiating *S. pneumoniae* and *S. mitis* (Kilian et al. 2008).

1.1.2 Clinical Disease

S. pneumoniae is the second commonest bacterial cause of death worldwide, and causes a wide range of diseases from pneumonia, meningitis, otitis media and septicaemia (Musher 1992; Coffey et al. 1998) (Fig 1.1). *S. pneumoniae* is part of the normal nasopharyngeal flora, and it is estimated that 40% or more of children carry *S. pneumoniae* in the upper respiratory tract, and carriage in adults ranges from 5 to 70%, depending on various environmental factors (Austrian 1986; Wu et al. 1997). Currently *S. pneumoniae* accounts for two-thirds of the cases with community acquired pneumonia (CAP) with known aeitology, and two thirds of the cases of bacteraemia (Fine et al. 1996). In the developing world an estimated 2.6 million children under 5 years of age die annually as a result of pneumonia, with *S. pneumoniae* being the single most common causative pathogen (WHO 2007). All disease states can lead to migration of *S. pneumoniae* from the site of infection into the bloodstream, resulting in pneumococcal sepsis (Gray et al. 1986). Furthermore, *S. pneumoniae* is an important cause of primary septicaemia in children in the developing world (Scott et al. 1996).

Patients particularly susceptible to pneumococcal infections include infants, the elderly, and those who are immunocompromised (either due to HIV infection or medical treatment through immunosuppression or splenectomy) or have certain chronic conditions such as diabetes, alcohol abuse or chronic lung disease. Prior respiratory infection with influenza virus is a major risk factor for pneumococcal infection, and smokers are also more susceptible to pneumonia (Jones et al. 1984; Nuorti et al. 2000; Dowell et al. 2003). Interestingly, males tend to be more likely to contract invasive pneumococcal disease (IPD) (Robinson et al. 2001). The highest disease rates occur in children between 11 months and 6 years old, and there is also increasing incidence of IPD in over 65 year olds, with a rate of approximately 75/100,000 (CDC 2000; Sleeman et al. 2001). Mortality rates exceed 20% are higher in the elderly and in young children range from 2% in developed countries to over 20% in the developing world (Plouffe et al. 1996; Robinson et al. 2001; Shibl et al. 2009).

The clinical importance of *S. pneumoniae* infections is compounded by increasing antibiotic resistance amongst clinical isolates (Jacobs 2004; Farrell et al. 2007). The first penicillin resistant strain was isolated in 1967, and resistance to anti-pneumococcal antibiotics including macrolides and β-lactams is increasing in both developed and developing countries. Recent studies estimated the worldwide prevalence of penicillin-resistant and macrolide-resistant *S. pneumoniae* ranged from 18·2 to 22·1% and from 24·6% to 31·8% (Felmingham 2002; Jacobs et al. 2003) respectively. The most prevalent serotypes resistant to penicillin and erythromycin include 6A, 6B, 9V, 14, 19A, 19F and 23F (Whitney et al. 2000). Furthermore, the disease burden of *S. pneumoniae* is increased by HIV infection, which is associated with both increased incidence of disease and by an

increasing rate of antibiotic resistance in *S. pneumoniae* strains (McEllistrem et al. 2002).

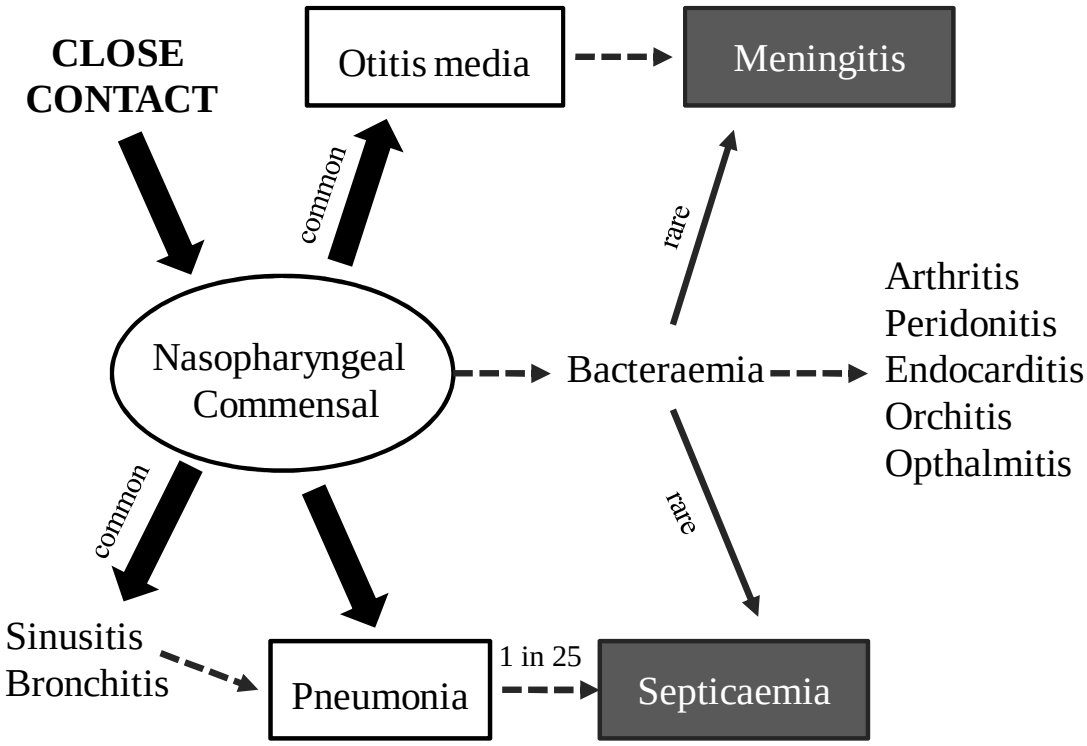


Fig 1.1 Clinical disease of *S. pneumoniae*

Transition from a carriage state to one in which symptomatic disease occurs is due to aspiration of bacteria from the nasopharynx into the lungs leading to pneumonia. Alternatively *S. pneumoniae* invade through the nasopharyngeal mucosa into the blood causing bacteraemia. If *S. pneumoniae* crosses the blood-brain barrier meningitis occurs. Mortality rates associated with *S. pneumoniae* disease are currently: otitis media, 0%; meningitis, 30%; septicaemia, 20%; pneumonia, 5%; sinusitis and bronchitis, 0%. Current estimates suggest 10% of adults and 50% of infants have nasopharyngeal colonisation with *S. pneumoniae*.

1.2 MOLECULAR EPIDEMIOLOGY

1.2.1 Biochemical Serotyping

It is essential for epidemiology studies that accurate and specific identification of *S. pneumoniae* occurs in patients suspected of having IPD. *S. pneumoniae* is surrounded by a polysaccharide capsule which is present on all strains which are isolated from patients with IPD. 91 antigenically different capsular types have been described, being distinguished by chemical differences in the type-specific capsular polysaccharides that can be identified by the specificity of type-specific antibody raised in rabbits (Henrichsen 1995). The 91 different antigenic types are divided into 46 serotype groups, as a certain degree of antigenic cross reactivity occurs (Fig 1.2). Mixture of the bacteria with antiserum causes the capsule to swell on binding of homologous antibody. This determines the basis of the standard test known as the Quellung Reaction, in which the preparation is stained with India Ink to visualize the swollen capsule zone using microscopy. Different specificities of antisera are available, from omniserum which recognizes all *S. pneumoniae* strains to serum which differentiates between the very similar 6A and 6B serotypes (Statens Serum Institute). However, the Quellung Reaction requires skillful microscopic examination and the recent introduction of molecular typing methodologies offers an alternative method of identifying capsular serotype.

Type	Antigenic Formula	Type	Antigenic Formula
1	1a	19C	19a, 19c, 19f, 7h
2	2a	20	20a, 20b, 7g
3	3a	21	21a
4	4a	22F	22a, 22b
5	5a	22A	22a, 22c
6A	6a, 6b	23F	23a, 23b, 18b
6B	6a, 6c	23A	23a, 23c, 15a
6C	6a, 6b	23B	23a, 23b, 23d
7F	7a, 7b	24F	24a, 24b, 24d, 7h
7A	7a, 7b, 7c	24A	24a, 24c, 24d
7B	7a, 7d, 7e, 7h	24B	24a, 24b, 24e, 7h
7C	7a, 7d, 7f, 7g, 7h	25F	25a, 25b
8	8a	25A	25a, 25c, 38a
9A	9a, 9c, 9d	27	27a, 27b
9L	9a, 9b, 9c, 9f	28F	28a, 28b, 16b, 23d
9N	9a, 9b, 9e	28A	28a, 28c, 23d
9V	9a, 9c, 9d, 9g	29	29a, 29b, 13b
10F	10a, 10b	31	31a, 20b
10A	10a, 10c, 10d	32F	32a, 27b
10B	10a, 10b, 10c, 10d, 10e	32A	32a, 32b, 27b
10C	10a, 10b, 10c, 10f	33F	33a, 33b, 33d
11F	11a, 11b, 11e, 11g	33A	33a, 33b, 33d, 20b
11A	11a, 11c, 11d, 11e	33B	33a, 33c, 33d, 33f
11B	11a, 11b, 11f, 11g	33C	33a, 33c, 33e
11C	11a, 11b, 11c, 11d, 11f	33D	33a, 33c, 33d, 33f, 6a
11D	11a, 11b, 11c, 11e	34	34a, 34b
12F	12a, 12b, 12d	35F	35a, 35b, 34b
12A	12a, 12c, 12d	35A	35a, 35c, 20b
12B	12a, 12b, 12c, 12e	35B	35a, 35c, 29b
13	13a, 13b	35C	35a, 35c, 20b, 42a
14	14a	36	36a, 9e
15F	15a, 15b, 15c, 15f	37	37a
15A	15a, 15c, 15d, 15g	38	38a, 25b
15B	15a, 15b, 15d, 15e, 15h	39	39a, 10d
15C	15a, 15d, 15e	40	40a, 7g, 7h
16F	16a, 16b, 11d	41F	41a, 41b
16A	16a, 16c	41A	41a
17F	17a, 17b	42	42a, 20b, 35c
17A	17a, 17c	43	43a, 43b
18F	18a, 18b, 18c, 18f	44	44a, 44b, 12b, 12d
18A	18a, 18b, 18d	45	45a
18B	18a, 18b, 18e, 18g	46	46a, 12c, 44b
18C	18a, 18b, 18c, 18e	47F	47a, 35a, 35b
19F	19a, 19b, 19d	47A	47a, 43b
19A	19a, 19c, 19d	48	48a
19B	19a, 19c, 19e, 7h		

Fig 1.2 Type designations and antigenic formulae of 91 types of *S. pneumoniae* The antigenic formulas represent arbitrary designations of cross-reactions as seen by the capsular reaction (Kauffmann et al. 1940). Adapted from Henrichsen, 1995

1.2.2 Molecular Typing Methods

Accurate characterization of *S. pneumoniae* is essential for molecular epidemiology, and traditional serotyping methodologies are not necessarily discriminating enough. Recently Multilocus sequence typing (MLST) has been developed for *S. pneumoniae* and has been very useful in characterising the epidemiology and ecology of the pneumococcus. MLST assigns numbers to alleles at housekeeping gene loci by nucleotide sequencing. These housekeeping genes are shikimate dehydrogenase (aroE), glucose-6-phosphate dehydrogenase (*gdh),* glucose kinase (*gki),* transketolase (*recP*), signal peptidase I (*spi*), xanthine phosphoribosyltransferase (*xpt*) and D-alanine-D-alanine ligase (*ddl*). Different polymorphisms within the nucleotide sequence of 450-500 bp internal fragments of these 7 housekeeping genes are assigned an allele number creating a 7 figure code that defines each sequence type (ST). MLST has been validated in *S. pneumoniae*, and there is a strong correlation between serotype and genetic relatedness in invasive *S. pneumoniae* (Enright et al. 1999). Most serotypes are composed of a limited number of ST strains but one ST may have different capsular serotypes.

Furthermore since MLST defines isolates based on their allelic profiles, it is possible to examine clustering between isolates and identify isolates with close or identical allelic profiles. As isolates diverge they accumulate single nucleotide differences in the 7 MLST loci, and relationships between isolates can become obscured by relatively minor recombination exchanges in these housekeeping genes. However MLST is sufficiently discriminatory to determine if isolates of *S. pneumoniae* from IPD in a geographical area

are the same strain or from different strains and how closely related strains causing disease in one geographic area are to those isolated from IPD world-wide.

Molecular typing methods have shown that a serotype of *S. pneumoniae* is normally comprised of several divergent genotypes. Changes in serotype can occur through recombination at the capsular locus (Coffey et al. 1998; Coffey et al. 1999; Ramirez et al. 1999), creating strains with the different capsular serotypes but the same ST. In addition, molecular serotyping techniques may also provide insights into multiple carriage, which has been difficult until recently due to technical challenges with conventional serotyping methods (Huebner et al. 2000; Brito et al. 2003; Lawrence et al. 2003).

1.2.3 Epidemiology of *S. pneumoniae*

There are at least 40 serogroups of *S. pneumoniae* which are potentially pathogenic and possibly all are, but only a relatively small number of these groups accounts for the majority of IPD in children worldwide (Hausdorff et al. 2000). There are geographic and age-related differences in the incidence of *S. pneumoniae* serotypes, and certain *S. pneumoniae* serotypes are recovered from IPD more frequently than others and have higher disease prevalence. In the USA, serogroups 4, 6, 9, 14, 18, 19 and 23 cause 80-90% of invasive disease, and in Europe the same serotypes cause 61-81% of invasive disease in children. However, most childhood pneumonia cases occur in the developing world, especially India, China, and Pakistan, whilst Bangladesh, Indonesia and Nigeria also have a high incidence of cases requiring hospitalization (Rudan et al. 2008). However it is likely

that some of the data from some countries is confounded by sampling issues and weak analytical technique, especially within Africa. A recent study in India found the most common serogroups isolated from IPD to be 1, 6, 19, 7, 5, 15, 14, 4, 16, and 18, giving a 71% serotype coverage from the 9-valent vaccine (INCLEN 1999). However, in Bangladesh the most common causative serogroups isolated are 2, 1, 14, 5, 7F, 45 and 12A which means the nine-valent vaccine will only have a 35.6% coverage (Saha et al. 2009). Interestingly, serotypes which tend to be associated with nasopharyngeal prevalence (eg 19 and 24) are highly prevalent in North America and Europe, whereas the invasive serotypes are more common in developing countries (Scott et al. 1996). Furthermore there are both increase carriage and higher levels of multiple carriage in the developing world (Greenwood 1999; Obaro et al. 2002)

In older children and adults there are a larger number of *S. pneumoniae* serotypes which are responsible for IPD. These data are confounded by an extremely wide age range of subjects and methodological differences between studies making meta-analysis unfeasible. However, serogroups 14, 4, 1, 3, 6 and 19 seem to cause a considerable proportion of IPD in this age group, and this is not limited by geographical area (Hausdorff et al. 2000). HIV infected patients in Africa were more likely to be have IPD caused by serotypes 6, 14, 19 and 23 compared to HIV negative individuals with IPD (Crewe-Brown et al. 1997). However in the USA, HIV positive individuals IPD was most likely due to serotypes 6A, 6B, 9N, 9V, 18C, 19A, 19F and 23F and this patients showed reduced incidence of IPD from serotype 1, 7F and 12F compared to non HIV infected individuals with IPD (Fry et al. 2003).

1.2.4 Invasive disease potential

The incidence of different *S. pneumoniae* serotypes isolated from IPD in developed countries has been used to make vaccine preparations. However, the prevalence of a given serotype of *S. pneumoniae* recovered from IPD does not necessarily take into account differences in exposure levels to that serotype. For example, serotypes which have a low potential to cause disease but are highly prevalent in the geographical area (leading to greater exposure) may lead to a larger proportion of IPD when compared to a serotype which is highly invasive but which is only rarely found colonising the nasopharynx. *S. pneumoniae* carriage is common in children under 5 years old, but IPD is a relatively rare event and recently several studies have been conducted examining the relationship between carriage prevalence and disease incidence in different populations. Calculation of an empirical odds ratio to compare the probability of invasive disease caused by a serotype has enabled the quantification of the invasive disease potential of different serotypes of *S. pneumoniae* (Brueggemann et al. 2003). Simply, this is a comparison between the frequency of IPD being caused by a given serotype with the frequency of carriage of that serotype in the population expressed as an odds ratio (OR). Serotypes 1, 4, 7F, 18C and 14 are consistently found to have a high OR for invasive disease potential whereas serotypes 6A, 6B, 23F, 9V, and 3 are among those found to be relatively poor at causing IPD (Brueggemann et al. 2003; Sandgren et al. 2004; Hanage et al. 2005). Hence although serotype 6B causes a considerable disease burden, in relation to its carriage levels, serotype 6B is less likely to cause IPD than serotype 14.

Furthermore, as these isolates were analyzed using MLST it becomes possible to calculate the invasive potential of individual strains or clones, even within a capsular serotype. Interestingly, invasive isolates are found to be consistently less genetically diverse than those isolated from carriage (Hanage et al. 2005), possibly suggesting a selection pressure on certain genes. In the studies based in Oxford and Finland there were no statistically significant differences in the invasive potential of different strains within the same capsular serotype (Brueggemann et al. 2004; Hanage et al. 2005). However, strains from the same serotype but which were genetically distinct were found to have a different OR for invasive potential in a Swedish study (Sandgren et al. 2004). In addition, some isolates which had differing capsular serotypes but which belonged to the same clone were found, and these strains were found to have the same disease potential (Sandgren et al. 2004). Other studies have also suggested that disease properties associated with the particular clonal type, as well as the capsular serotype, may contribute to the ability of *S. pneumoniae* to cause disease (Sjostrom et al. 2006). Furthermore a study on serotype 6 isolates from different geographical areas found that some STs were more associated with invasive disease and some were more associated with colonizing strains (Robinson et al. 2001).

1.2.5 Possible explanations for differences in *S. pneumoniae* strains disease potential

Given that it has been well established that a limited number of capsular serotypes are responsible for most IPD, there is surprisingly little data which would explain why this might be (Hausdorff et al. 2000). Colonization of the nasopharynx is thought to be essential for the development of IPD, and data from infants suggests both that IPD can occur shortly after exposure to *S. pneumoniae* and that IPD may develop some time after successful colonization (Austrian et al. 1977; Gray et al. 1986). It has been suggested that increased prevalence of a serotype or strain of *S. pneumoniae* would lead to an increased exposure to that particular serotype of clone and hence increased IPD would be result. This may be the case for serotypes such as 6B, which have a high carriage rate and an associated high frequency of disease but are hence relatively poorly invasive based on molecular epidemiology studies. Host and bacterial factors which maintain patterns of nasopharyngeal carriage are poorly understood, although recently it was suggested that the biochemical structure of the polysaccharides could affect their degree of encapsulation and hence interactions with host immunity (Weinberger et al. 2009).

However, the molecular epidemiology studies have clearly shown that carriage prevalence does not fully explain why some serotypes of *S. pneumoniae* are more invasive than others. The polysaccharide capsule (CPS) is widely presumed to be the most important virulence factor for *S. pneumoniae*. Hence changes in the structure of the polysaccharides and degrees of encapsulation could affect the ability of a given strain to invade host tissue, evade immune mechanism and may even potentially explain why some serotypes cause particular disease states more frequently than others. There is a great degree of genetic

variation between strains of *S. pneumoniae*, with genome variation of up to 10% of between strains of different serotypes and 2% between clones of the same serotype (Hakenbeck et al. 2001). Since different clones of the same serotype can have different invasive potentials (Sandgren et al. 2004) non-capsular factors must also contribute to invasive disease potential. Therefore the presence, absence and allelic variation in virulence factors such as CbpA, pneumolysin, PspA as well as the biochemical structure of the cell wall could influence invasive disease potential, but the relative contribution of each of these factors compared to the effect of the capsule is unknown. Furthermore, it remains unclear as to which host-bacterial interactions are important influences on the invasive potential of different *S. pneumoniae* strains and capsular serotypes.

1.2.6 Effects of vaccines on *S. pneumoniae* ecology

Current vaccine preparations utilize the antigenic nature of CPS to induce immunity in a given individual. The 23 valent (Pneumovax II) polysaccharide vaccine (serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F) has various drawbacks including a poor induction of immune responses in children under 2 years, the elderly and immunocompromised patients. Pneumovax does not significantly affect carriage of *S. pneumoniae* in adults and children (MacLeod et al. 1945; Dagan et al. 1996). A 7-valent polysaccharide vaccine (Prevenar) (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F) uses polysaccharide from these 7 capsular serotypes conjugated to diphtheria toxoid in order to induce a switch from thymus-independent to thymus-dependent response, inducing a T-cell response. Prevenar shows an efficacy of 97.4% against IPD caused by vaccine serotypes and the CDC has reported that the incidence of IPD caused by vaccine serotypes

has decreased by 94% (CDC 2008). Conjugate vaccines have a significant impact on pneumonia, as well as a reduction in asymptomatic carriage of vaccine serotypes (Obaro et al. 1996; Black et al. 2002). The 9-valent pneumococcal conjugate vaccine reduces carriage rates of vaccine serotypes by up to 50% in South Africa and a study from the US found that only 3% of strains isolated from carriage were of vaccine serotypes (Mbelle et al. 1999; Huang et al. 2009). However, whilst Prevenar is an effective vaccine in children under 5 years, non-vaccine serotypes have increased in prevalence in vaccinated populations (Dagan et al. 1996; Obaro et al. 1996). Huang et al recently demonstrated that carriage of non-vaccine serotypes rose from 15% in 2000 to 29% in 2007, with common non-vaccine serotypes including 19A (16%), 6A (12%), 15B/C (11%), 35B (9%), and 11A (8%) (Huang et al. 2009). In particular serotype 19A has emerged as increasing in prevalence in vaccinated populations (Kyaw et al. 2006; Singleton et al. 2007). Serotype replacement is due mainly to expansion of existing strains of non-vaccine serotypes, although vaccine escape by capsular switching also occurs, in which a vaccine serotype strain acquires the *cps* locus of a non-vaccine serotype by recombination (Beall et al. 2006; Brueggemann et al. 2007). The clinical effect of serotype replacement is not fully known; there seems to be an increase in prevalence of non-vaccine serotypes causing acute otitis media (Eskola et al. 2001; O'Brien et al. 2003) but data on IPD is equivocal. Recently increases in non-vaccine serotypes causing IPD in Alaska (Singleton et al. 2007) and throughout the USA have been reported (CDC 2008), with other studies finding no evidence of serotype replacement (Black et al. 2004; Black et al. 2006; Black et al. 2007).

1.3 CELLULAR IMMUNITY TO PNEUMOCOCCUS

1.3.1 Neutrophils

Neutrophils (PMNs) are polymorphonuclear cells which are the first immune cells recruited to sites of inflammation from the blood. They are terminally differentiated from haematopoietic stem cells in bone marrow and have a life span which encompasses only a few hours. Upon contact with a microbe, neutrophils engulf the pathogen in a phagosome which fuses with intracellular granules containing antimicrobial peptides such as α-defensins, cathepsin G, lysozyme, lactoferrin and reactive oxygen species to form a phagolysosome. Bacteria are killed in the phagolysosome by a combination of non-oxidative mechanisms and the respiratory burst, which forms a varied set of reactive oxygen species (ROS) (Klebanoff 2005). The transmembrane and cytosolic subunits of the large NADPH-oxidase complex assemble at the phagosomal membrane and transfer electrons to molecular oxygen producing superoxide (O_2^-). O_2^- reacts to H_2O_2 spontaneously or through catalysis by superoxide dismutase. H_2O_2 acts as a substrate of myeloperoxidase to form hypochlorous acid (which is the most bactericidal oxidant in neutrophils) in addition to hypobromous and hypoiodous acid (Hampton et al. 1998). In addition to the intracellular killing mechanisms, PMNs release granule proteins and chromatin forming fibres called neutrophil extracellular traps (NETs). The major components of the NETs are DNA and associated histones H1, H2A, H2B, H3 and H4. NETs contain smooth stretches of 15–17 nm in diameter which consist of naked DNA and globular domains of around 25nm in which there is chromatin. The granule proteins from azurophilic, specific and gelatinase granules which are found in NETs and the histones are all bactericidal (Hirsch 1958). Neutrophils phagocytose microbes within minutes, and it is

thought that NETs are formed after phagocytic killing mechanisms are exhausted since NET formation initiates after *in vitro* infection (Urban et al. 2006).

The importance of neutrophils for the host immune response is seen in patients who are neutropenic and these individuals show increased incidence of IPD. PMNs are one of the first cells recruited to sites of *S. pneumoniae* infection and colonisation (Dallaire et al. 2001; van Rossum et al. 2005). It has also been demonstrated that a *S. pneumoniae* strain which normally causes only asymptomatic carriage will become invasive when neutrophils are depleted from mice (Matthias et al. 2008). Furthermore patients with chronic granulomatous disease, in which there is reduced ROS generation, have increased bacterial infections although interestingly they do not show a particular increase in IPD incidence. PMNs isolated from these patients kill *S. pneumoniae* as efficiently as neutrophils from healthy individuals, and recent data has suggested that PMNs kill *S. pneumoniae* through serine proteases (Kaplan et al. 1968; Standish et al. 2009). This is compatible with an increased incidence of bacterial infection in patients with Chedaki-Higashi syndrome, who have a deficiency in levels of microbicidals and reduced granule mobilization (Root et al. 1972; Ganz et al. 1988). Neutrophil NETs are also capable of binding *S. pneumoniae* although the capsule may prevent NET mediated killing (Wartha et al. 2007).

Mouse models of pneumonia indicate that *S. pneumoniae* are found within neutrophils (Bergeron et al. 1998), however data on the role of PMNs for immunity to *S. pneumoniae* pneumonia is equivocal. Impaired PMN recruitment leads to poor control of *S. pneumoniae* pneumonia in mice (Nakasone et al. 2007; Sun et al. 2007), but other results suggest that

disease progresses less rapidly in neutropenic mice (Marks et al. 2007). In addition excessive PMN activity may lead to lung damage and neutrophil recruitment causes many features of lung disease through the release of oxidants and proteinases (Henson et al. 1987; Weiss 1989). *In vitro* phagocytosis of *S. pneumoniae* by PMNs is complement dependent (Yuste et al. 2008) and neutrophils require complement to clear *S. pneumoniae* colonization from the nasopharynx (Lysenko et al. 2005; Yuste et al. 2008). Although complement factors are present in BALF and increase during *S. pneumoniae* infection (Robertson et al. 1976; Kerr et al. 2005) the contribution of complement to the interactions of *S. pneumoniae* with neutrophils during the development of pneumonia is not clear. Certainly neutrophils are important in systemic immunity to pathogens, with *S. pneumoniae* being no exception (Wang et al. 2002). Complement-mediated neutrophil phagocytosis is the dominant host immune mechanism against *S. pneumoniae* in systemic infection and the classical pathway is the major complement pathway responsible for clearance of *S. pneumoniae* from systemic infection (AlonsoDeVelasco et al. 1995; Brown et al. 2002).

1.3.2 Alveolar Macrophages

AMs are the resident specialised phagocytic cell in the lung and are early effectors of innate immune responses against *S. pneumoniae*. AMs are relatively long lived cells which are derived from blood borne monocytes although some AM replication occurs (Thomas et al. 1976; van oud Alblas et al. 1979). Recently a brisk turnover of resident AMs and replacement of these cells with newly recruited monocytes was demonstrated in *S. pneumoniae* pneumonia (Taut et al. 2008). An important role for AMs in preventing *S. pneumoniae* pneumonia is suggested by the increased incidence of pneumonia in subjects

exposed to cigarette smoke or welding fumes, which impair macrophage activity, and by experimental data showing that depletion of AMs increases *S. pneumoniae* replication within the lung in mice (Dockrell et al. 2003). In addition, impaired function of AMs allows increased *S. pneumoniae* replication within the lung and a higher mortality in animal models of pneumonia (Arredouani et al. 2004; Arredouani et al. 2006; Didierlaurent et al. 2008). Hence avoidance of AM-mediated clearance from the lung is probably critical in dictating whether inhalation or aspiration of a pathogen results in lung infection.

AMs are able to phagocytose *S. pneumoniae*, but this is hindered by the capsule and effective opsonisation with complement, antibody and other opsonins is required (Jonsson et al. 1985). AMs are efficient at killing internalised pneumococci, however as bacterial numbers increase this process becomes overwhelmed (Gordon et al. 2000; Dockrell et al. 2003). Macrophages increase nitric oxide levels following challenge with *S. pneumoniae* and NADPH oxidase has also been implicated in macrophage killing (Kerr et al. 2004; Marriott et al. 2004). Depletion of AMs has been shown to reduced survival to several lung pathogens highlighting their importance for pulmonary immunity (Broug-Holub et al. 1997; Kooguchi et al. 1998; Cheung et al. 2000; Traeger et al. 2009). AMs are an important source of proinflammatory cytokines (such as TNFα) which acts as a chemoattractant for neutrophils and are also able to phagocytose apoptotic PMNs and hence mediate resolution of the inflammation (Fig 1.3) (Knapp et al. 2003). Depletion of AMs leads to reduced clearance of apoptotic neutrophils and lead to increased levels of the anti-inflammatory IL-10 and AM depleted mice showed reduced survival compared to control mice (Knapp et al. 2003).

AMs express various receptors which may be involved in the recognition, phagocytosis and the innate activation which is induced following *S. pneumoniae* infection. These include but are not limited to FcR (fragment, crystallizable receptor); complement receptor 1 and 3 (CR1 and CR3); scavenger receptors (SR) such as SR-A and macrophage receptor with collagenous structure (MARCO); Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN); toll-like receptors 2 and 4 (TLR2 and TLR4); and dectin-1 (Taylor et al. 2005). $MARCO^{-/-}$ mice show impaired pulmonary clearance of *S. pneumoniae*, increased inflammation and reduced survival (Arredouani et al. 2004). Furthermore the association of macrophages with *S. pneumoniae* is significantly impaired in $MARCO^{-/-}$ deficient macrophages, suggesting this receptor is important in innate immune responses against pneumococcus (Arredouani et al. 2004). SR-A I/II deficiency has also been shown to cause impaired *in vivo* phagocytosis of *S. pneumoniae*, reduced lung clearance and increased pneumonic inflammation as well as reduced survival (Arredouani et al. 2006). However the relative contribution of each of these receptors to the function of AMs and the pathogenesis of pneumococcal disease remains unclear. Furthermore how pneumococcal virulence factors including CPS modulate their function is not fully understood.

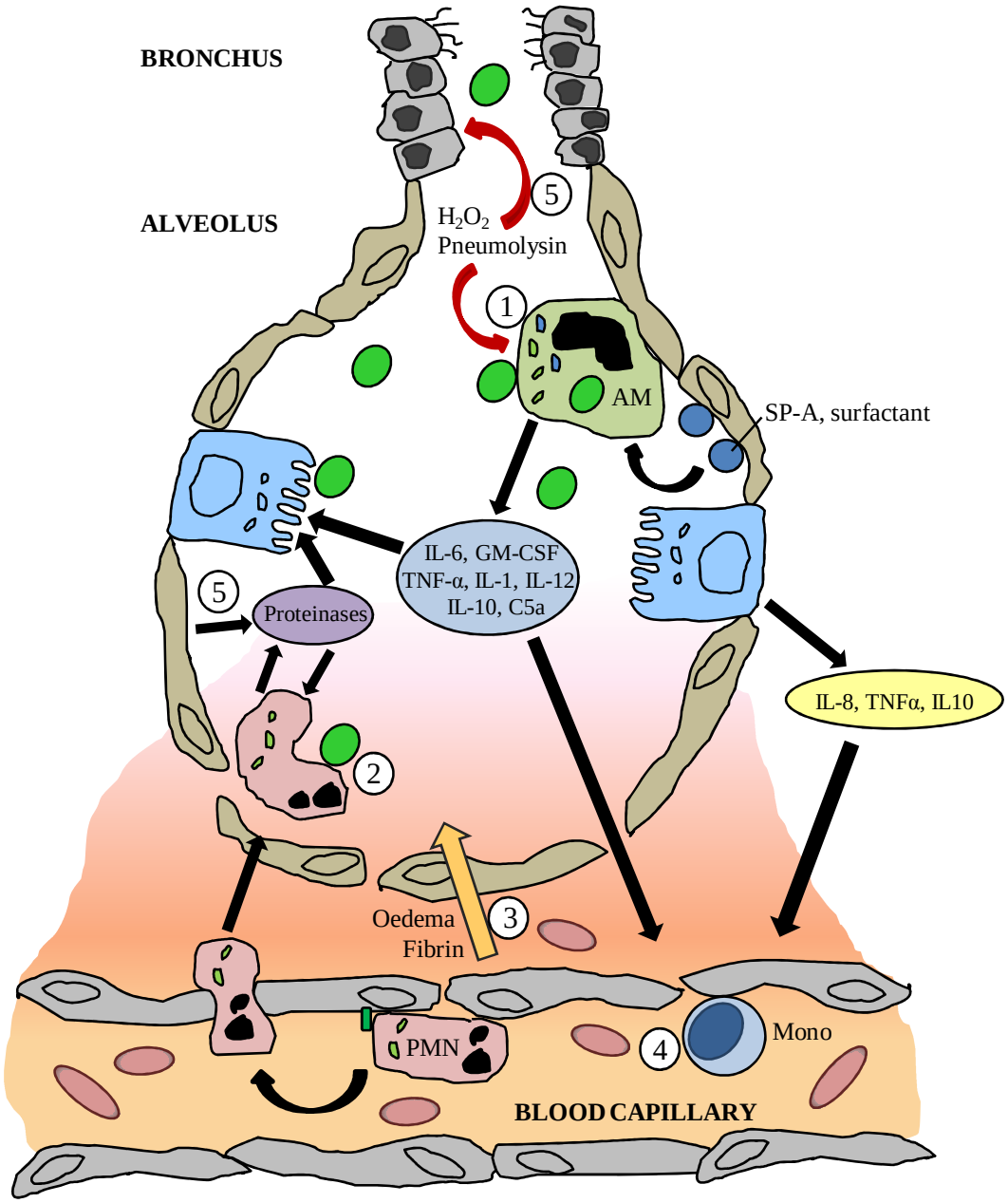


Fig 1.3 Role of AMs and PMNs in *S. pneumoniae* lung infection

1. AMs are the resident phagocytic cell in the lung, killing *S. pneumoniae* (green ovals) and releasing important pro- and anti-inflammatory cytokines to modulate the immune response. 2. PMNs are recruited to the site of infection by chemokines including IL-8 and C5a. 3. As the infection progresses the epithelium becomes leaky, and the alveolus fills with oedema and fibrin. 4. Monocytes do not respond until late in *S. pneumoniae* pneumonia. 5. Pneumolysin and hydrogen peroxide contribute directly to pulmonary damage, killing epithelial cells, macrophages and arresting ciliary beating. Proteinases are secreted by AMs and epithelium and include elastase, matrix metalloproteinases, α_1-proteinase inhibitor and α_2-macroglobulin and act to kill bacteria.

Recently data suggests that induction of apoptosis in AMs by bacterial infection may be a host mediated strategy (Zychlinsky et al. 1997; Dockrell et al. 2001; Ali et al. 2003). AM apoptosis induced by *S. pneumoniae* shows features of mitochondrial apoptosis and interestingly there seems to be no effect of death receptors including Fas ligands or the TNFα receptor on this process (Dockrell et al. 2001). Apoptotic AMs are phagocytosed by non-apoptotic AMs, and this is associated with decreased neutrophil recruitment and reduced TNFα levels, potentially defining a mechanism by which apoptosis may regulate lung inflammation (Marriott et al. 2006; Marriott et al. 2008).

Systemic clear of *S. pneumoniae* is dependent on an intact reticuloendothelial system and *S. pneumoniae* are found within the liver and spleen in sepsis (Brown et al. 1981; Brown et al. 1981; Brown et al. 1983). The macrophages found within the spleen are important in systemic immunity to *S. pneumoniae*, and marginal zone macrophages express a SIGN R1 receptor which binds and internalises CPS (Mitchell et al. 1983; Evans 1985; Kang et al. 2004).

1.3.3 Dendritic Cells

Dendritic cells (DCs) are the most important antigen-presenting cell, whose function is to ingest and present antigen to T lymphocytes. Tissue DCs engulf antigens at infection sites, become activated as part of the innate immune response, migrate to lymphoid tissue and differentiate into highly effective antigen-presenting cells (Janeway et al. 2002). Hence DCs act as a critical bridge between innate and adaptive immunity, initiating adaptive immune responses (Banchereau et al. 2000). Mature DCs are distinguished by co-

stimulatory molecules which act in synergy with antigen in the activation of T lymphocytes, and there are also changes in surface expression of MHC, adhesion molecules, and cytokine production in mature antigen presenting cells (Reis e Sousa 2001). The capsule polysaccharide significantly impairs *S. pneumoniae* phagocytosis by DCs, and the serotype 1 capsule polysaccharide has been shown to activate CD4+ T cells through presentation of the polysaccharide by MHC class II-positive tubules of murine DCs (Stephen et al. 2007; Noske et al. 2009). It is likely that *S. pneumoniae* induces apoptosis in DCs, which seems to occur through both a rapid caspase-independent but pneumolysin-dependent as well as a delayed pneumolysin-independent mechanism, and this may be a survival strategy for the bacteria since apoptotic DCs do present function as efficient antigen presenting cells (Colino et al. 2003; Kleindienst et al. 2003).

1.3.4 T lymphocytes

T lymphocytes are thymus matured cells composed of different subsets known and helper (T_H) $CD4^+$, cytotoxic (T_C) $CD8^+$, regulatory, memory and gamma-delta T cells. T_H immune responses are divided into three classes referred to as T_H1, T_H2 and T_H17. T_H1 responses typically promote cell-mediated killing of pathogens, and are primarily made in response to bacteria which stimulate macrophages or NK cells and viruses. T_H1 T cells produce lymphotoxin, TNF-β and IFN-γ which acts to activate macrophages. This induces increased microbial killing in phagolysosomes, release of inflammatory cytokines and promotes T_H1 cell differentiation. In contrast the T_H2 response is directed against extracellular pathogens, allergens and toxins and tends to induce a cytokine response composed of IL-4 and IL-13. These cytokines stimulate B cells to produce antibody and mast cells to release

inflammatory mediators. The cytokine milieu determines whether a naive T cell develops into a T_H1 or T_H2 cell. The mutual antagonism of the cytokine products of each T_H response further supports this T cell differentiation. T_H17 lymphocytes produce IL-17, inducing stromal cells to produce inflammatory cytokines including IL-6, IL-8 and IL-22.

T cells have been implicated in the host immune response to *S. pneumoniae* since patients with MHC I and (due to mutations in TAP-1 or TAP-2) II deficiencies who have 'bare lymphocyte' syndrome show increased incidence of *S. pneumoniae* pneumonia (Klein et al. 1993; Donato et al. 1995; Carneiro-Sampaio et al. 2007). A T_H1 response to pneumococcus is protective in human *S. pneumoniae* infection (Kemp et al. 2002). Furthermore $CD4^+$ deficient mice are more susceptible to infection and show increased bacterial counts in both the lung and in blood (Kadioglu et al. 2000).

Recently the role of $CD4^+$ T lymphocytes in *S. pneumoniae* disease has been examined further. There is a rapid influx of $CD4^+$ T cells into infected areas of lung observed (Kadioglu et al. 2004). However this response is abrogated in the absence of pneumolysin and studies performed *in vitro* have confirmed that the $CD4^+$ chemotaxis is dependent on pneumolysin (Kadioglu et al. 2000; Kadioglu et al. 2004). Furthermore these cells appear to be required for efficient nasopharyngeal clearance of *S. pneumoniae* and intranasal immunization does not protect $CD4^+$ deficient mice, indicating a role for these cells in nasopharyngeal immunity (Malley et al. 2005; van Rossum et al. 2005). T_H17 $CD4^+$ cells have been shown to mediate the recruitment to the nasopharynx of monocytes and macrophages as well as neutrophils in response to *S. pneumoniae*, in a mechanism which is

independent of IFNγ and IL-4 may depend upon TLR2 (Lu et al. 2008; Zhang et al. 2009). T_H17 $CD4^+$ cells may also have a role in inducing the production of anti-microbial peptides by epithelial cells in response to *S. pneumoniae* (Liang et al. 2006).

Classically T_C cells are involved in host immunity against intracellular pathogens and viruses, and act to induce the death of host cells which have become infected by programming apoptosis in target cells. This occurs through release from the T_C cell of preformed granules which contain membrane disrupting proteins such as perforin, serine proteases, cathepsin and stored effector molecules including Fas ligand. $CD8^+$ T_C cells have been implicated in lung immunity to *S. pneumoniae* (Angrill et al. 2001) and these cells are essential for antibody-mediated pulmonary protection against *S. pneumoniae*, but do not seem to be required for systemic immunity (Tian et al. 2007). $CD8^+$ T cells may mediated an antibody dependent effect through their production of IFNγ, which has been shown to be an important chemokine for immunity to *S. pneumoniae, Klebsiella pneumoniae* and *Cryptococcus neoformans* (Rubins et al. 1997; Moore et al. 2002; Lindell et al. 2005). Interestingly $CD8^+$ T cells may in fact suppress antibody responses to CPS, suggesting T lymphocytes are important regulators of the response to CPS (Jeurissen et al. 2002).

In addition NK T cells are a lymphocyte population which express both T cell receptors and NK cell markers and act in innate immune responses producing IFNγ and IL-4 (Taniguchi et al. 2000; Kronenberg et al. 2002). Mice lacking NK cells have been shown to have increased *S. pneumoniae* load in the lungs and reduced survival compared to control mice (Kawakami et al. 2003). These NK T cells have been linked to a MCP-1 dependent early

pulmonary immune mechanism which promotes PMN recruitment (Kawakami et al. 2003).

1.3.5 B lymphocytes and antibody

B lymphocytes are cells which play a key role in humoral responses by producing antibody and developing into memory B cells. Follicular B cells systemically re-circulate and mount antibody responses against thymus dependent protein antigens, whereas marginal zone and $CD5^-$ B cells generate thymus independent antibody responses (Mond et al. 1995). Marginal zone, CD5+ and CD5- B cells collaborate to provide immunity to *S. pneumoniae* (Haas et al. 2005). In addition there seems to be a distinct B cell populations which produce IgM ($CD19^+CD27^+IgM^+IgD^+$ memory B cells) and another which produces IgG ($CD19^+CD27^+IgM^-IgD^-$ switched memory B cells) to both pneumococcal polysaccharide and protein antigens (Moens et al. 2008).

Patients with SCID have increased incidence of IPD after passive transfer of maternal antibodies ceases to provide protection against *S. pneumoniae* (Carneiro-Sampaio et al. 2007). Further evidence of the importance of antibodies in host immunity to *S. pneumoniae* is provided by individuals with X-linked agammaglobulinaemia and specific IgG2, IgA and selective anti-polysaccharide antibody deficiencies as well as those with hyper IgM type-2 and IgE syndrome, who all show increased susceptibility to *S. pneumoniae* infections (French et al. 1995; Hammarstrom et al. 2000; Conley et al. 2002; Winkelstein et al. 2003; Quartier et al. 2004; Grimbacher et al. 2005).

Antibody responses specific for protein and polysaccharide antigens are induced by distinct mechanisms (Mond et al. 1995). CPS antigen stimulates B cells independently of MHC class II T lymphocytes by inducing crossing linking of the B cell receptors, and antibody titres do not rise beyond the level induced after primary immunization. T_H cells are involved in regulation of this response, which is thought to be mediated through an essential interaction between the transiently expressed CD40 ligand on T_H cells which activates B cells through cell surface expressed CD40 (Jeurissen et al. 2002). CPS stimulates terminally differentiated B cells and therefore does not induce responses in young children, and elicits antibodies that are isotype restricted to IgM and IgG_2 and to a lesser extent IgG_1 in humans (Casal et al. 2003). In contrast, antibodies against sub-capsular proteins are T cell dependent, requiring B cells to be activated by a $CD4^+$ T cell which is also specific for the antigen which the responding B cell has recognised and presented (linked recognition). B cells stimulated in this way can proliferate, differentiate into plasma cells and can generate memory B cells. A wide range of cell wall and membrane associated pneumococcal proteins can generate a protective antibody responses (Kalin et al. 1987; Berry et al. 1989; Crain et al. 1990; Talkington et al. 1996; Brooks-Walter et al. 1999; Ferreira et al. 2006; Moens et al. 2008). It is hoped that antibodies generated against essential pneumococcal proteins could provide immunity against multiple serotypes in vaccines. However, proteins such as CbpA have a varied structure between strains, and hence antibodies generated against proteins on one *S. pneumoniae* strain may not confer complete protection against another strain. Furthermore it remains unclear what the relative contribution of antibodies against non-capsular pneumococcal antigens is for natural immunity to *S. pneumoniae*, compared to the contribution of anti-capsular antibody.

Current vaccines against *S. pneumoniae* use the polysaccharide capsule to induce a protective antibody response. Unconjugated polysaccharide vaccines (such as Pneumovax) fail to induce a sustained memory response from the host immune system, and the immune response to Pneumovax is thymus independent. This response is typically predominated by IgM and repeated immunisation does not induce a higher level of anti-capsular antibody in the host (Schneerson et al. 1980). In contrast, conjugate vaccines such as Prevenar stimulate thymus dependent response, resulting in germinal centre formation and the induction of immunological memory (Borriello et al. 1997; Garside et al. 1998). Many prominent B and T cell membrane proteins play a significant role in the formation and stabilization of antigen-specific B-cell-T-cell conjugates (Clark et al. 1991; Sharpe 1995). CD40 to CD40L and B7 to CD28 interactions co-stimulate cell activation and proliferation (Clark et al. 1991; Sharpe 1995). Data has shown that the major co-stimulatory pathway involving the ligation of CD28 on T cells with B7 molecules on activated B cells is critical for the induction of immunologic memory to capsule polysaccharide and for isotype switching to IgG in the response to conjugate vaccines (Guttormsen et al. 1999).

An antibody response from the host is not limited to IPD, and nasopharyngeal colonisation induces a serum IgG and secretory IgA response against PspA of the inoculated strain (McCool et al. 2002). However antibody does not seem to promote clearance of *S. pneumoniae* from the nasopharynx, as antibody levels of PspA in both serum and nasal lavages positively correlate with disease severity in mice, and immunodeficient mice that respond poorly to protein antigens or which are unable to produce antibody clear colonisation as well as wild-type mice (McCool et al. 2004). However, antibody plays an important role in systemic immunity to *S. pneumoniae* and this is thought to occur through

the opsonic action of antibody (Robbins et al. 1995; Lefeber et al. 2003; Martinez et al. 2006). However it is worth noting that IgG and IgM antibody can activate the complement system through the classical pathway, and some of the effect of antibody may be mediated by complement (Heidleberger et al. 1942; Brown et al. 1982; Brown et al. 1983).

Natural IgM antibodies are germ-line coded and not affinity maturated antibodies, which have a genetically limited antigen repertoire and only few mutations expand this range. However, a certain acquired genetic variability of the innate immunoglobulin receptors is achieved by combinatorial association of germ-line immunoglobulin genes (Vollmers et al. 2006). Additional mutations, deletions and additions in recombination events guarantee a variability which is sufficient to cover a wide antigen range on pathogenic organisms and gives a adequate protection by these antibodies (Constantinescu et al. 1997; Rothenberg 2000; Vollmers et al. 2006). Peritoneal cavity $CD5^+$ B cells are believed to produce natural IgM antibodies, and these cells are precursors of $CD11b/CD18^-$ splenic IgM Ab-secreting cells (van Rooijen 1989; Kawahara et al. 2003). The repertoires of natural IgM antibodies may be driven by selection by self-antigens or by exposure to microorganisms (van Rooijen 1989; Berland et al. 2002). Natural IgM has an important role in innate immune defence mechanisms and are involved in early recognition of pathogens and cancer cells (Ben-Aissa-Fennira et al. 1998; Boes 2000; Ulvestad et al. 2001; Brandlein et al. 2003). Furthermore, natural IgM has been shown to contribute to early immunity against encapsulated bacteria, leading to clearance of bacteria from the blood to the spleen and provide specific protections against *S. pneumoniae* (Brown et al. 2002; Baxendale et al. 2008).

1.3.6 Inflammation

Both humoral and cellular immune responses are regulated by cytokines, which are produced by many cell types including AMs. However cytokines are a double edged sword and a balance needs to be maintained between pro- and anti-inflammatory cytokines. This is particularly important for the lung, where gas exchange is severely impaired by inflammation and excessive inflammation would be detrimental to the host. TNFα, IL-1, IL-6 and IL-8 levels rise quickly in areas of lung infected with *S. pneumoniae* (Dehoux et al. 1994; van der Poll et al. 1996; van der Poll et al. 1997). In addition AMs from individuals infected with *S. pneumoniae* produce G-CSF which is likely to contribute to PMN release from bone marrow (Tazi et al. 1991). Mice treated with an anti-TNFα antibody when infected with *S. pneumoniae* have increased bacterial CFU isolated from the lungs and rapidly develop fatal disease (van der Poll et al. 1997). IL-6 has also been shown to be protective in lung infection in mouse models using a similar model (van der Poll et al. 1997). However the relationship between cytokine profiles and clinical outcome in pneumonia patients remains unclear, with some studies finding no relationship between TNFα levels and outcome and others showing increased TNFα levels correlate with less severe disease (Marik et al. 1993; Moussa et al. 1994; Puren et al. 1995)

1.3.6.1 Receptors that initiate inflammation

The inflammatory immune response is an essential component of the host immune response to pathogens, and there are various receptors through which this response is mediated. One class of receptor which is important in regulating inflammatory responses to bacterial pathogens but not phagocytosis is the Toll like receptor family. TLRs play a crucial role in the host immunity to invading microbes by recognizing pathogen-associated molecular patterns (Takeda et al. 2003). They are members of the interleukin-1 receptor (IL-1R) superfamily and are characterized by the presence of a leucine-rich repeat domain in their extracellular regions and a Toll/IL1 receptor (TIR) domain in the intracellular regions (Takeda et al. 2003). Most TLRs share a common signalling pathway via the adaptor molecule, myeloid differentiation primary-response protein 88 (MyD88) (Takeda et al. 2003). Once stimulated TLRs recruit MyD88 and the death domain of MyD88 then binds the death domain of IL1R-associated kinase (IRAK). The signal is then propagated via TNF receptor-associated factor-6 (TRAF6), leading to the activation of NFκB and MAP kinases, and the transcription of immunologically relevant genes (Takeda et al. 2003; Akira et al. 2004). A second TIR containing adaptor protein, TIR-associated protein (TIRAP)/MyD88-adaptor-like (Mal), is involved in the MyD88-dependent pathways of TLR1/2, TLR2/6 and TLR4, but not other TLRs (Horng et al. 2002; Yamamoto et al. 2002). The most important TLR-expressing cell types are thought to be DCs, macrophages, and B cells (Iwasaki et al. 2004). *S. pneumoniae* has been shown to induce NFκB activation through TLR2 and 9, and the ability to activate TLR2 may involve recognition of the cell wall components peptidoglycan and lipoteichoic acid (Yoshimura et al. 1999; Moore et al. 2003; Mogensen et al. 2006). However, this data needs to be re-evaluated in the light of recent data showing that TLR2 stimulation by *Staphylococcus aureus* is mediated entirely through lipoproteins,

demonstrating that previous work illustrating the effect of lipoteichoic acid was due to contamination of the cell wall preparations with lipoproteins (Hashimoto et al. 2006; Hashimoto et al. 2006; Schmaler et al. 2009). There is also some evidence that pneumolysin is able to stimulate cells through TLR4, although some investigators finding no evidence of TLR4 activation (Yoshimura et al. 1999; Malley et al. 2003; Branger et al. 2004; Mogensen et al. 2006).

Another subset of pattern recognition receptors is the nucleotide-binding oligomerization domain (NOD) proteins, and these receptors are involved in the intracellular recognition of pathogens. NOD1 recognizes peptidoglycans containing meso-diaminopimelate acid found mainly in Gram-negative bacteria (Girardin et al. 2003), and is expressed by most cells. In contrast, NOD2 mediates responsiveness to muramyldipeptide MurNAc-L-Ala-D-isoGln which is conserved in peptidoglycans of most bacteria and the expression of NOD2 is restricited to leukocytes, DCs and epithelial cells (Ogura et al. 2001; Inohara et al. 2003). NOD2 activates the NFκB signaling pathway as well as the p38 and ERK1/2 MAPK pathways, and NOD1 has been shown to activate the JNK pathway (Girardin et al. 2001; Kobayashi et al. 2005). It has been shown that *S. pneumoniae* is recognized by NOD2 and signal transducing molecules including IRAK, IRAK2, TRAF6 and NIK are involved in NFκB activation by NOD proteins in cells stimulated with pneumococci (Opitz et al. 2004).

In contrast, the inflammasome is a multiprotein complex which is responsible for the activation of caspases 1 and 5, which results in the production and release of the pro-inflammatory cytokines IL-1β and IL-18 (Martinon et al. 2002). The central proteins

involved in inflammasomes are NOD-like receptors with pyrin domains (NALPs) although little is known about their precise function. The stimuli which induce assembly and activation of the inflammasome, although it is thought that the inflammasome is activated through the recognition of pathogen-associated molecular patterns by leucine rich repeats found on NALP proteins. Intracellular pathogens including *Shigella* and *Salmonella* have been shown to induce apoptosis in infected macrophages, which occurs through caspase 1 (Monack et al. 2001; Obregon et al. 2003).

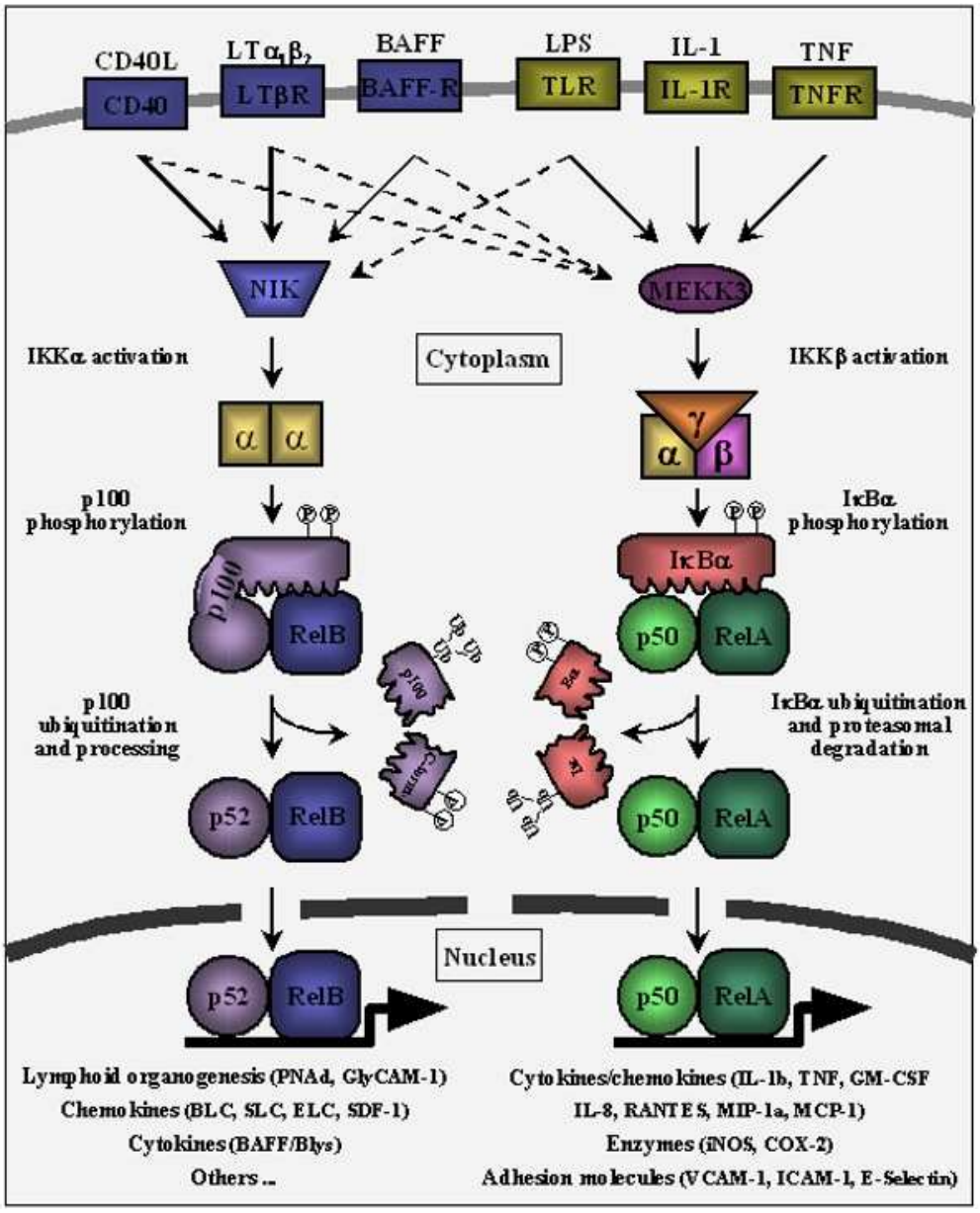
Another important cell surface-receptor involved in the regulation of the inflammatory response is the C5a receptor (CD88). The C5aR is expressed on the surface of immune cells including macrophages, neutrophils and T cells. C5aR primarily couples to Gαi2 (Skokowa et al. 2005) which is a pertussis toxin-sensitive G protein, although ectopically expressed C5aR and C5aR in some cells including monocytes can also couple to Gα16 (Kalant et al. 2003), a pertussis toxin-insensitive G protein. Through these G proteins the C5aR is able to activate the Ras/Raf/MAP kinase signalling pathway (Gerard et al. 1994). The C5aR has been shown to mediate chemotaxis, superoxide generation and the release of histamine, interleukins, leukotrienes and enzymes from granules from granulocytes as well as upregulation of adhesion molecules (Goetzl et al. 1974; Hartman et al. 1981; Morita et al. 1989; Elsner et al. 1994). C5 has been demonstrated to have a significant effect on PMN recruitment to the lung of mice infected with *S. pneumoniae* as well as other pathogens (Snyderman et al. 1971; Toews et al. 1984; Winkelstein 1984; Rubins et al. 1995). C5a also improves efficiency of PMN phagocytosis.

It is likely that all of these receptors contribute to inflammation in pneumococcal disease, although the effect of the capsule on cell activation through these pathways remains unclear.

1.3.6.2 The NFκB transcription factor pathway

Inflammation involves a sequential release of mediators, leukocyte recruitment to the inflammatory site and activation of recruited leukocytes. This response is self-limiting and both the induction and resolution of inflammatory responses seem to be regulated by transcription factors including the NFκB transcription factor pathway. NFκB activation is tightly regulated by signals that degrade IκB, which classically occurs through phosphorylation by the IκB kinase (IKK) complex followed by degradation by the 26S proteosome (Karin et al. 2000). The IKK complex is composed of catalytic subunits known as IKKα and IKKβ as well as a regulatory subunit IKKγ (Nishikori 2005). The key NFκB family member in classical NFκB activation is RelA, which is found in a wide variety of cells including macrophages (Moine et al. 2000; Roux et al. 2004). The classical pathway is typically activated by a ligand binding to TNF receptors, TCRs, BCRs, or the TLR-IL1 receptor superfamily, leading to increased transcription of genes encoding for chemokines, cytokines including TNFα, IL-8 and RANTES as well as adhesion molecules, cyclooxygenase 2 and iNOS (Vane et al. 1994; Hobbs et al. 1999; Karin et al. 2000; Tak et al. 2001; Yamamoto et al. 2001) (Fig 1.4). Classical pathway activation tends to result in enhanced inflammatory responses and promotes cell survival. In contrast, the alternative pathway is activated through certain TNF receptor family members including CD40, CD30, B cell activating factor belonging to the TNF family receptor and the lymphotocin β

receptor (Nishikori 2005). The alternative pathway NFκB2 and p100 subunits are phosphorylated at two C-terminal sites by the IKKα homodimer and then ubiquinated, which leads to targeting of the inhibitory C-terminus for proteosomal degradation (Nishikori 2005). Activation of the alternative pathway typically regulates development of lymphoid organs and adaptive immune mechanisms (Fig 1.4). Only the NFκB subunits RelA and p50 have been identified in extracts of lungs exposed to bacteria, suggesting that pulmonary NFκB signalling to pneumococcus would be mediated through the classical pathway (Mizgerd et al. 2002; Jones et al. 2005). Furthermore patients with deficiencies in IRAK4 (IL-1 receptor associated kinase) and NEMO-dependent NFκB activation have a marked increased in incidence of IPD (Ku et al. 2005). Furthermore common polymorphisms in the IκB genes *NFKBIA* and *NFKBIE* genes are associated with increased frequency of *S. pneumoniae* infection (Chapman et al. 2007). AMs have an essential role in NFκB activation in the lungs (Mizgerd et al. 2002). RelA deficiency decreases cytokine expression, PMN migration and *S. pneumoniae* killing in the lung following intratracheal infection (Quinton et al. 2007). IκBα is degraded in both mouse lungs and human bronchial epithelial cells exposed to *S. pneumoniae*, and inhibition of IκB kinase complex blocks cytokine release from epithelial cells, suggesting that epithelial cells are an alternative pro-inflammatory cytokine source to AMs in *S. pneumoniae* pneumonia (Schmeck et al. 2004).



<u>Fig 1.4</u> Activation of NFκB by the alternative and classical pathways

Signalling through TNFR, IL-1R or TLRs activates the classical NFκB pathway involving predominantly the α and β subunits of the IKK complex. Nuclear translocation and DNA-binding of p50-RelA occurs through phosphorylation of IκBα and ubiquitin-dependent proteasomal degradation. Membrane-bound $LT\alpha_1\beta_2$ heterodimers, CD40, and BAFF activate via their respective receptors the kinases NIK and IKK. Phosphorylation of p100 results in the processing of the precursor to the p52 subunit and nuclear accumulation of p52-RelB heterodimers. There is significant cross talk as signalling through the LTβR also results in the induction of RelA complexes and LPS can also trigger the processing of p100 to p52.

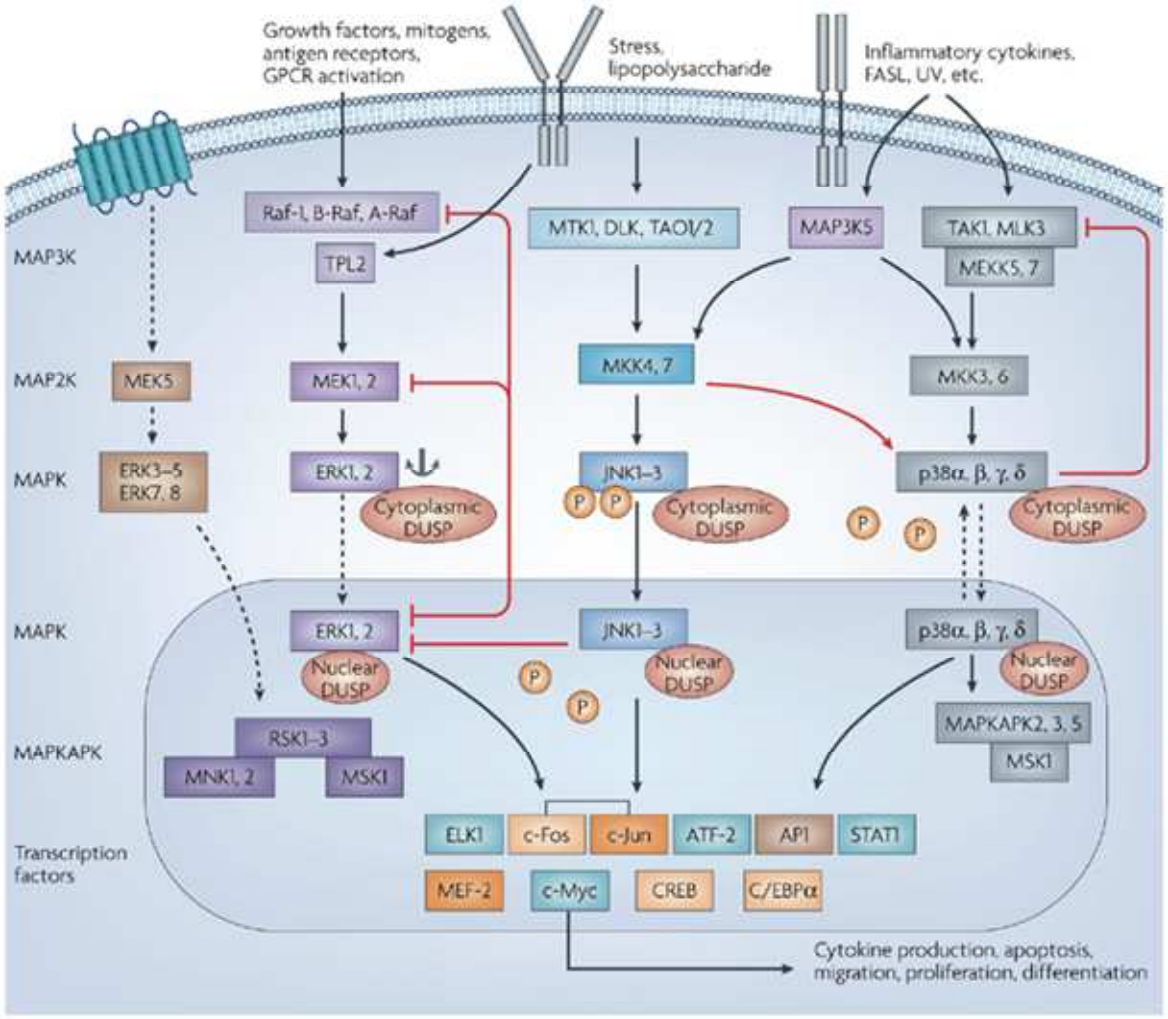
1.3.6.3 The MAPK pathways

Since pneumococcal components can activate Toll like receptors (TLRs) it is also likely that macrophage responses to *S. pneumoniae* are affected by mitogen activated protein kinases (MAPKs). MAPKs affect several macrophage properties including pro- and anti-inflammatory cytokine production, apoptosis, cell migration and particle uptake which are all important in pulmonary immune responses to *S. pneumoniae* (Hazzalin et al. 2002; Blander et al. 2004; Yates et al. 2005; Shin et al. 2008). MAPK cascade pathways are composed of a MAPK, MAPK kinase (MAPKK) and a MAPKK kinase (Guha et al. 2001; Jeffrey et al. 2007). To date 5 distinct groups of MAPKs have been characterised: extracellular signal-related kinases 1 and 2 (ERK 1/2); c-Jun animo-terminal kinases (JNKs); p38 isoforms α, β, γ and δ; ERKs 3 and 4; and ERK 5 (Roux et al. 2004) (Fig 1.5). MAPK pathways can be activated through TLRs and NOD, which are important in immune responses to pneumococcus (Wellmer et al. 2002; Barton et al. 2003; Koedel et al. 2004; Opitz et al. 2004). However, the role of the precise pathways involved and the effect of specific virulence factors on signalling pathways remains unclear. In addition is it unknown if and consequently how CPS modulates these responses, since studies were carried out using only encapsulated *S. pneumoniae* without comparison to unencapsulated strains.

1.3.6.4 The ERK pathway

Many studies have shown that LPS can activates the ERK 1/2 pathway in monocytes and macrophages. The ERK 1/2 kinase domains have a Threonine-Glutamic acid-Tyrosine (TEY) motif which is activated by phosphorylation (Guha et al. 2001). Dominant-negative repressors of Ras and c-Raf inhibit LPS induction of the TNFα promoter in RAW 264.7

macrophages, suggesting that activation of tyrosine kinases leads to activation of the MEK-ERK 1/2 pathway in a Raf-1-dependent mechanism (Geppert et al. 1994; Arbibe et al. 2000). Inhibition of MEK reduces production of inflammatory cytokines including IL-1, IL-8, TNFα following LPS stimulation of monocytes (Scherle et al. 1998). Upon stimulation, ERK 1/2 accumulates in the nucleus but activated ERK 1/2 phosphorylate numerous substrates including membrane proteins, nuclear substrates (SRC-1, NF-AT, Elk-1, c-Myc, c-Fos, STAT3 and Pax6), cytoskeletal proteins and MAPK-activated protein kinases (Chen et al. 2001) (Fig 1.5). ERK pathways have been shown to induce iNOS and TNF production during LPS and IFNγ stimulation of murine macrophages (Ajizian et al. 1999). Recently a novel pneumococcal virulence factor EstA was shown to stimulate RAW 264.7 macrophages via the ERK pathway which led to iNOS production (Kang et al. 2009).



<u>Fig 1.5</u> The MAPK signalling pathways

The three main MAPK signalling pathways ERK, JNK and p38 mediate immune cell functional responses through multiple receptors. MAPK cascade pathways are composed of a MAPK, MAPK kinase (MAPKK) and a MAPKK kinase (Guha et al. 2001; Jeffrey et al. 2007). ERK 1/2 accumulates in the nucleus but activated ERK 1/2 phosphorylate numerous substrates including membrane proteins, nuclear substrates (SRC-1, NF-AT, Elk-1, c-Myc, c-Fos, STAT3 and Pax6), cytoskeletal proteins and MAPK-activated protein kinases. p38 activates transcription factors including activating transcription factor-2, Elk-1, Gadd153, myocyte enhancer factor 2C and Sap1a as well as phosphorylating downstream kinases. Red arrows indicate feedback or cross talk within the pathways. Taken from Jeffrey et al, Nature Reviews Drug Discovery, 2007.

1.3.6.5 The p38 pathway

p38 was identified in LPS-stimulated macrophages and this pathway includes isoforms of p38/stress activated protein kinase-2/reactive kinase (Han et al. 1994; Zhang et al. 2000). Cdc42, PAK and Rac1 are upstream signalling molecules which activate the p38 pathway. MAPK kinases involved in activation of the p38 cascade include MKK3 and MKK6 and possibly MKK4 (Derijard et al. 1995; Raingeaud et al. 1996). Furthermore disruption of the *mkk3* gene causes a selective defect in p38 activation and TNFα induction of cytokine gene expression (Wysk et al. 1999). p38 activates transcription factors including activating transcription factor-2, Elk-1, Gadd153, myocyte enhancer factor 2C and Sap1a as well as phosphorylating downstream kinases which regulate gene expression by phosphorylating transcription factors such as cAMP response element binding and activating transcription factor 1 (Rolli et al. 1999; Guha et al. 2001) (Fig 1.4). p38 may impact on the NFκB transcription factor pathway through phosphorylation of the p65 subunit after IL-1β or thrombin stimulation or bacterial infection (Madrid et al. 2001; Schmeck et al. 2004). *S. pneumoniae* infection has been shown to lead to p38 pathway activation in epithelial cells, which resulted in NFκB and RNA polymerase II recruitment to the *cox2* promotor (N'Guessan et al. 2006).

1.3.6.6 The JNK pathway

This pathway is activated in RAW 264.7 macrophages through innate immune mechanisms and contains the c-Jun N-terminal kinase (JNK) (Hambleton et al. 1996; Karin 1998). There are 2 isoforms of JNK which phosphorylate the N-terminus of c-Jun. Upstream protein kinases including p21-activated kinase, germinal center kinase and hPAK1 activate the

JNK pathway (Bagrodia et al. 1995; Brown et al. 1996; Hirai et al. 1997). MEKK1, MEKK4 and MUK/DLK/ZPK also activate the JNK pathway in response to various inflammatory stimuli (Xia et al. 1998; Davis 1999; Tournier et al. 1999). In addition MKK4 and MKK7 are direct activators of JNK (Guha et al. 2001). Many of the downstream targets of JNK are transcription factors which regulate genes encoding inflammatory mediators, including activating transcription factor-2, Elk-1 and c-Jun (Karin et al. 1997) (Fig 1.5). JNK has been shown to be essential for the transcriptional activation of inflammatory cytokine genes in response to GBS but not *S. pneumoniae*, and in turn JNK activates activator protein-1 and NFκB, which are critical for activation of the TNF promoter (Kenzel et al. 2006). However, other studies show *S. pneumoniae* induces IL-8 expression by human epithelial cells which depends on JNK activation and recruitment of phosphorylated c-Jun to the *il8* promoter (N'Guessan et al. 2006; Schmeck et al. 2006).

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1.4 COMPLEMENT

1.4.1 Overview

The complement system is an example of a biochemical proteinase cascade, and although it is a component of innate immunity it also links to the adaptive immune system. The proteins and glycoproteins that comprise the complement system are synthesized by tissue macrophages, monocytes and epithelial cells of the genitourinary, gastrointestinal and respiratory tracts. These proteins circulate in the blood as inactive zymogens which are activated by proteases, although some components of the complement system are cell surface bound. Complement proteins are also present in bronchoalveolar lavage fluid (BALF) but at levels approximating 10% those found in serum (Kerr et al. 2005). There are 3 pathways known as the classical, mannose-binding lectin (MBL) and alternative pathways that activate the cascade (Walport 2001). All complement pathways result in deposition of C3 convertase on the pathogen cell surface, cleavage of C3 into C3a and C3b and subsequent C5 convertase formation and cleavage (Fig 1.6).

The classical pathway is activated when C1q binds directly to pathogenic surfaces, binds the Fc receptor of antibody complexed with antigen, or non antibody proteins like CRP and SAP (Hughes-Jones et al. 1979; Volanakis et al. 1981; Poon et al. 1985; Bristow et al. 1986). In addition a novel C-type lectin, SIGN-R1, was shown to bind CPS, capture C1q and activate the classical pathway (Kang et al. 2006). C1q binding leads to change in the conformation of the associated $(C1r:C1s)_2$ complex (Bauer et al. 1981), with cleavage of C1r and then C1s (Schumaker et al. 1987). This in turn leads to C4 cleavage, followed by

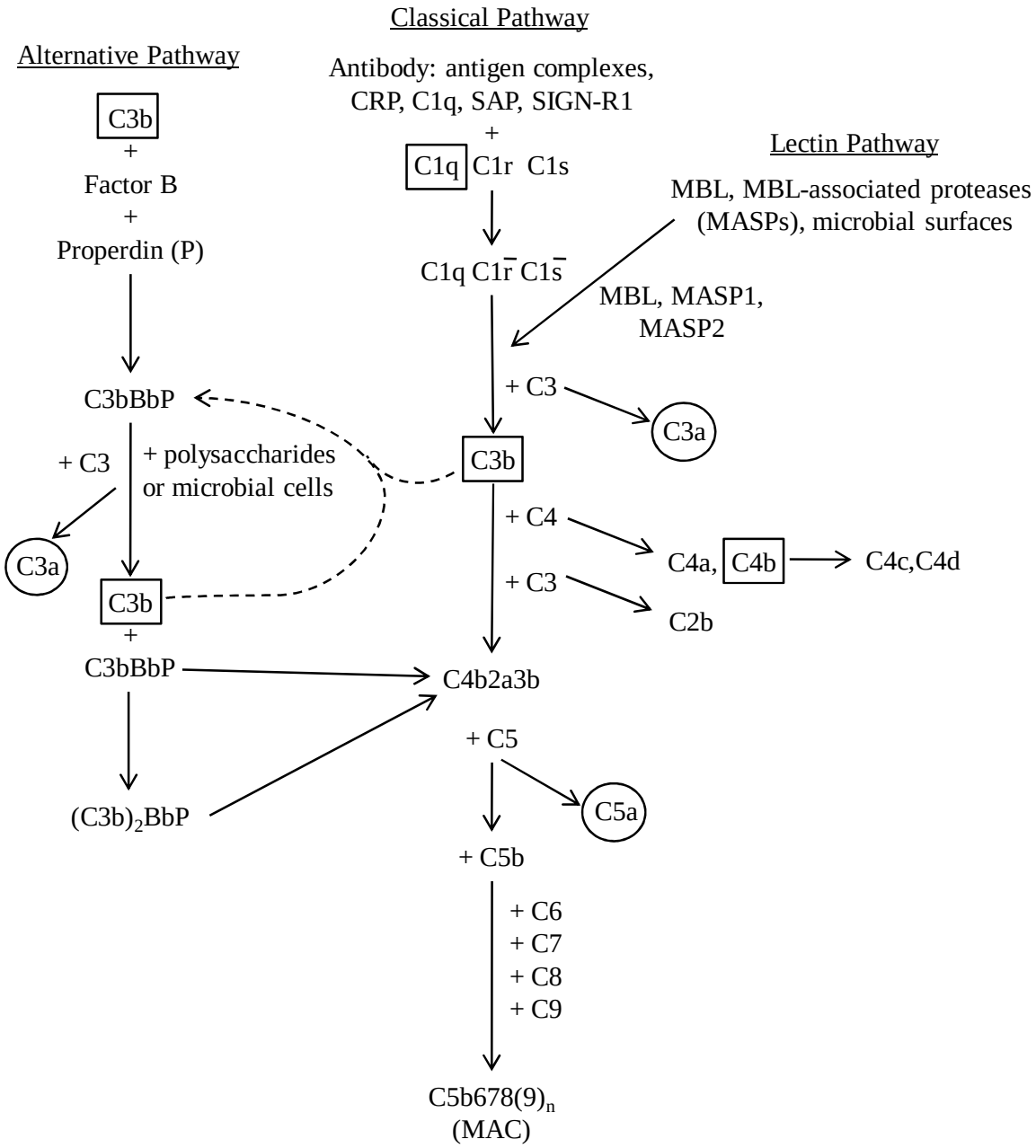


Fig 1.6 Schematic of the classical, MBL and alternative complement pathways

Factors which stimulate each pathway are shown in addition to the cascade sequence of each complement pathway. The MBL pathway interacts with the classical pathway to produce C1qC1rC1s and both pathways then proceed by the same route. The alternative pathway is shown to be separate from the classical pathway and only shares the late stages of the cascade with the classical cascade. The amplification loop of the alternative pathway is shown with a dashed arrow. All three pathways result in formation of the MAC. Anaphylaxins are highlighted with circles and opsonins with rectangles. Adapted from (Loeffler 2004).

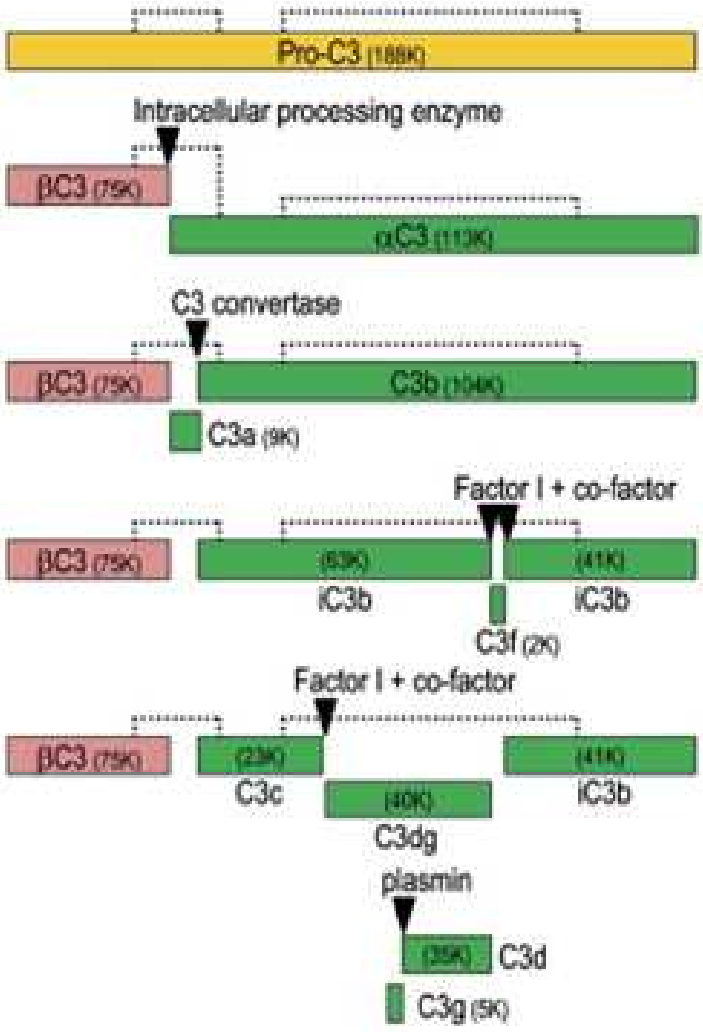
C2, resulting in formation of C4b2b on the target surface. The C4b2b acts as the C3 convertase of the classical pathway, and is covalently bound to the pathogen surface (Walport 2001). C4b is also an opsonin for phagocytosis. The classical pathway is regulated by C4b binding protein (C4BP) which dissociates C4b2b by dissociating the C2 from the complex (Hourcade et al. 1989). The MBL pathway is similar to the classical pathway, but mannose-binding lectin (MBL) replaces C1q and binds directly to sugar residues on the cell surface, specifically mannose residues (Hajela et al. 2002). When the MBL complex is bound to the pathogen surface MASP-2 is activated to cleave C4 and C2, and this initiates the subsequent steps of the classical pathway (Hajela et al. 2002).

In contrast, spontaneous C3 hydrolysis on the surface of pathogens initiates the alternative pathway. Hydrolysed C3 binds to factor B which is then cleaved by factor D into factor Bb, resulting in the formation of the alternative pathway C3 convertase, C3bBb (Walport 2001). The C3bBb complex deposits multiple C3b molecules on the pathogen surface, leading to opsonisation of the bacterium and MAC component activation (Walport 2001). On host cells C3bBb is regulated by FH, CR1 and DAF, which displace factor Bb from the complex and inactivate the C3 convertase (Hourcade et al. 2002). FH competes with factor B to displace Bb and binds preferentially to C3b which is already bound to cells due to its affinity for sialic acid residues (Fujita et al. 1999). The alternative pathway also increases the C3 convertase bound on the pathogen cell surface through an amplification loop (Fig 1.6)

The C3b formed by either the classical or alternative pathway and C5 convertase act to cleave C5 producing C5a and C5b (Vogt et al. 1978). C5a is a potent chemoattractant for neutrophils, monocytes and macrophages and enhances phagocytosis (Goldstein et al. 1974; Mollnes et al. 2002; Hawlisch et al. 2004). C5b initiates assembly of the later components of the complement cascade into the membrane attack complex (MAC). C5b first binds C6, and the complex then binds C7. The binding of C7 results in a conformation change in this protein thereby exposing a hydrophobic site, and the C5b67 complex inserts into the lipid bilayer of the cell membrane. C8 binds to the complex and also inserts into the membrane, and this is followed by the binding of multiple C9 molecules. Eventually 10-18 molecules of C9 polymerise to form a pore in the cell membrane, which allows free passage of solutes and water across the lipid bilayer of the bacterial cell, resulting in bacterial cell lysis (Walport 2001).

1.4.2 Conformational changes in C3 on activation

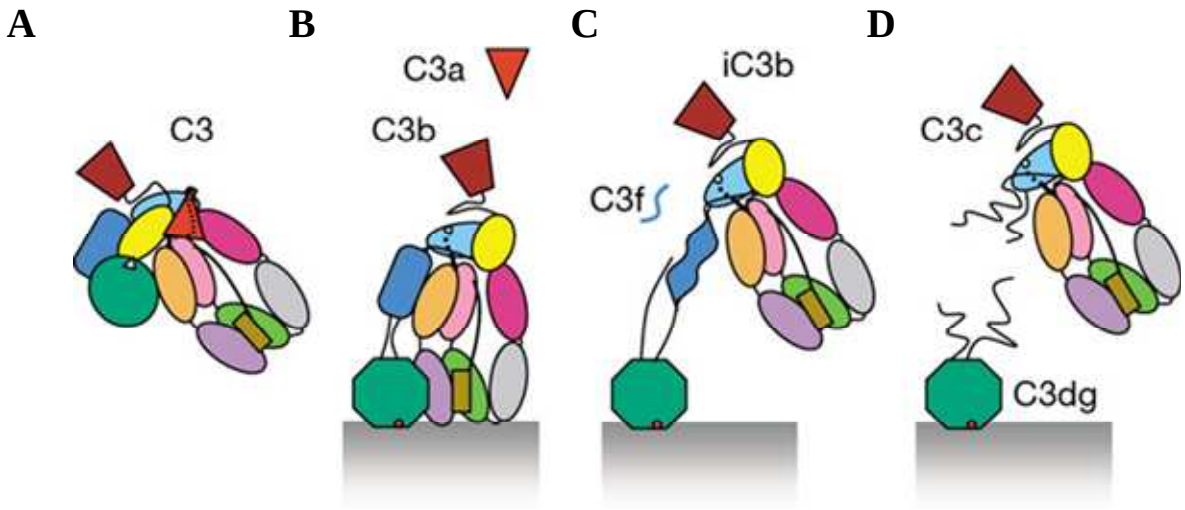
The central step in the complement cascade system is cleavage of C3 (186kDa) into C3b (177kDa) and C3a (9kDa). As this occurs C3b is split by Factor I and a co-factor in two positions to form iC3b (inactive C3b) and C3f. iC3b is cleaved further forming C3c and C3dg, and other proteases produce fragments including C3d and C3g (Fig 1.7). C3b formation is a tightly regulated step, as it is able to stimulate uptake of opsonised particles by phagocytes via complement receptor (CR) 1, 3, 4 and CRIg as well as upregulating B-cell response 10,000 fold through co-stimulatory and B-cell receptors (Dempsey et al. 1996; Carroll 2004; Helmy et al. 2006). The C3 β-ring provides a stable platform for the



<u>Fig 1.7</u> C3 degradation pathway

C3 is cleaved by an intracellular processing enzyme into βC3 and αC3. C3 convertase then cleaves αC3 into C3b, which is cleaved at two sites into iC3b by Factor I and a co-factor. Molecular weights shown are for human proteins. Taken from Kang et al, Cell 2006.

functionally important domains of the α-chain that undergo conformational rearrangements (Fig 1.8). Conversion of C3 into C3b involves proteolytic removal of a small anaphylatoxin domain inducing marked structural rearrangements in the protein, resulting in the exposure and activation of the thioester moiety that binds via a thioester bond to target surfaces. The C3 residues involved in the formation of a thioester bond are protected in C3 so that the reaction site is not exposed, but due to conformational changes that occur in conversion to C3b these residues become exposed and highly reactive towards hydroxyl nucleophiles (Janssen et al. 2007). Furthermore, the conversion of C3 also exposes binding sites of factor



<u>Fig 1.8</u> Schematic showing the conformational changes in the C3 activation pathway

(A) Unactivated C3, (B) C3b with C3a, (C) iC3b with C3f and (D) C3dg with C3c. These conformational changes determine the binding affinities towards soluble proteins (factor B, properdin and FH) and cell-surface receptors (eg CR1-4, CRIg, DAF and MCP) that underlie the biological activity of C3.

Taken from Janssen, Nature 2006.

B and properdin which stabilizes the resulting C3bBb convertase (Janssen et al. 2006). Binding of factor B to C3b (and subsequent cleavage of factor B by factor D) yields the short-lived C3bBb complex (Fishelson et al. 1984), which converts C3 into C3b and C3a, thereby amplifying the complement response and forming the $C3b_2Bb$ complex that cleaves C5 to initiate terminal complement components. Regulators of complement (FH, CR1 and DAF) dissociate the C3bBb complex through steric hindrance at their binding sites, and hence act as co-factors in the proteolysis of C3b into iC3b (Janssen et al. 2006).

1.4.3 Complement interaction with *S. pneumoniae*

Both clinical disease and animal models have indicated the importance of the complement in the host immune response to *S. pneumoniae* and the role of specific complement pathways in IPD. Patients with C2 deficiency show an increased incidence of IPD and serum from these patients has a reduced ability to opsonise *S. pneumoniae* with C3 resulting in decreased phagocytosis (Yuste et al. 2008). There is a CPS fixation pathway which activates the classical pathway and this pathway has been shown to be the most important in mouse *S. pneumoniae* infection models (Brown et al. 2002; Kang et al. 2006).

The role of the MBL pathway is contentious, with MBL deficient patients showing increased IPD incidence (although it remains unclear if this is a serotype specific phenomenon) but the MBL pathway has no effect on *S. pneumoniae* infection in mouse models (Brown et al. 2002; Roy et al. 2002). The classical pathway may be activated by antibody, SAP or CRP to targets on *S. pneumoniae* (Fig 1.9). SAP has been shown to bind

to structures on microbial surfaces including LPS, phosphorylcholine and mannose or galactose glycan residues and aids complement mediated immunity against *S. pneumoniae* (Hind et al. 1984; Schwalbe et al. 1992; de Haas 1999; Yuste et al. 2007). CRP also binds phosphorylcholine and activates the classical pathway through C1q, and furthermore binds capsule polysaccharide (Szalai et al. 1995). Both SAP and CRP exert their effects on the complement system by providing a C1q binding site, which then activates the classical complement pathway (Ying et al. 1993; Volanakis 2001). However, the direct binding of C1q to bacteria may also activate the classical pathway independently of antibody or pentaxrins (Clas et al. 1981; Alberti et al. 1993; Butko et al. 1999). The alternative pathway also contributes to host defence against *S. pneumoniae* with mice lacking factor D showing slowed kinetics in C3 opsonisation of pneumococcus and factor B deficient mice developing more rapidly progressing disease (Xu et al. 2001; Brown et al. 2002). The main role of the alternative pathway is thought to be amplification of C3b/iC3b deposited on the bacterial cell surface once complement activation has been initiated by the classical or MBL pathway (Walport 2001; Brown et al. 2002).

S. pneumoniae has various proteins that interact with complement and inhibit complement mediated immunity. These include CbpA, PspA, pneumolysin, phpA and phtD, and the effects of these proteins on complement are described below.

(a) CbpA

Choline binding protein (CbpA or PspC) is a predominant protein isolated in the choline binding proteins of *S. pneumoniae* (Rosenow et al. 1997), and is highly polymorphic between pneumococcal strains. CbpA is a 75 kDa multifunctional surface protein which is able to bind to secretory IgA, C3 and FH (Hammerschmidt et al. 1997; Smith et al. 2000; Dave et al. 2001). The differing CbpA structure has been shown to affect the binding of FH, which is a potent regulator of the alternative pathway (Quin et al. 2006). Theoretically CbpA could reduce alternative pathway activity by increasing the rate of C3 degradation to iC3b, causing dissociation of factor B (Bf) from the C3 convertase reducing C3b deposition, or inhibiting C3bBb formation by preferentially binding C3b. Furthermore, CbpA binds thioester-disrupted C3 which is not recognized by neutrophils as it is bound incorrectly (Cheng et al. 2000), thereby interfering with complement opsonisation of *S. pneumoniae*. However, although the affinity of CbpA for FH has been demonstrated, the effects of this interaction on immunity against *S. pneumoniae* are relatively poorly defined and different investigators have found conflicting results (Janulczyk et al. 2000; Dave et al. 2001; Iannelli et al. 2002). PspC increases iC3b and decreases C3b/iC3b deposition on D39 *S. pneumoniae*, however other results seem to indicate loss of PspC has little effect on total C3 deposition on pneumococcus (Lu et al. 2006; Li et al. 2007; Quin et al. 2007).

(b) PspA

Pneumococcal surface protein A (PspA) was the first surface exposed protein that was found to bind choline residues and varies in structure from CbpA in the amino acid sequence of the α-helical motifs (Holtje et al. 1975; Brooks-Walter et al. 1999;

Hollingshead et al. 2000). PspA has been shown to have a role in resistance to complement deposition and is found on almost all *S. pneumoniae* (Hollingshead et al. 2000; Ren et al. 2003). PspA is highly expressed by *S. pneumoniae* during infection and affects complement deposition through the alternative pathway, although the precise mechanism for this is unknown (Ogunniyi et al. 2002; Yuste et al. 2005). PspA varies in size between strains from 67-99 kDa and has a high polar electrostatic charge, which stabilizes the charge of the polysaccharide capsule (Jedrzejas et al. 2000). This electronegative charge on PspA might inhibit complement deposition and activation, with mutants that lack PspA cleared faster from the blood in systemic infection (McDaniel et al. 1987; Tettelin et al. 2001; Ren et al. 2003). Mice deficient in C3 or factor B are unable to clear mutants lacking PspA, whereas C5 deficient mice can clear the mutant strain (Tu et al. 1999). In addition, C3 serum levels are significantly reduced within 30 minutes of infection with a strain possessing PspA compared to a mutant strain lacking PspA (Tu et al. 1999). Furthermore mutant *S. pneumoniae* strains lacking both PspA and pneumolysin cannot spread from the lung into the blood in complement sufficient mice (Yuste et al. 2005). This paper suggests that C3 deposition is inhibited by PspA modulating the alternative pathway and pneumolysin inhibition of the classical pathway, which assists the establishment of *S. pneumoniae* septicaemia (Yuste et al. 2005).

(c) Pneumolysin

Pneumolysin is able to activate the classical pathway of the complement system in the absence of specific antibody (Paton et al. 1984; Mitchell et al. 1991). It was previously thought that pneumolysin was released into the serum by *S. pneumoniae* and activated complement by binding the Fc portion of IgG which lead to complement depletion in the serum (Mitchell et al. 1991). However Price and Camilli recently showed that pneumolysin is actually localised to the cell wall, and consequently its role complement interactions with *S. pneumoniae* needs to be reconsidered (Price et al. 2009). However, the complement activating property of pneumolysin has been shown to be associated with bacterial growth in both the lung and blood within 24 hours of intratracheal inoculation (Jounblat et al. 2003).

(d) Others

Pneumococcal histidine triad (Pht) proteins are a family of proteins which share extensive peptide sequence identity (Adamou et al. 2001). Currently the role of this protein family in pneumococcal virulence is poorly understood, however data seems to suggest that PhtB may cleave C3, based on fragments of these protein showing C3 degradation activity in human serum (Hostetter 1999; Zhang et al. 2001). Recently it was shown that removal of all four family members seems to be needed to reduce complement deposition on *S. pneumoniae*, and this may occur through recruitment of FH by these proteins (Ogunniyi et al. 2009). No doubt in the future more surface proteins will be identified that affect complement activity.

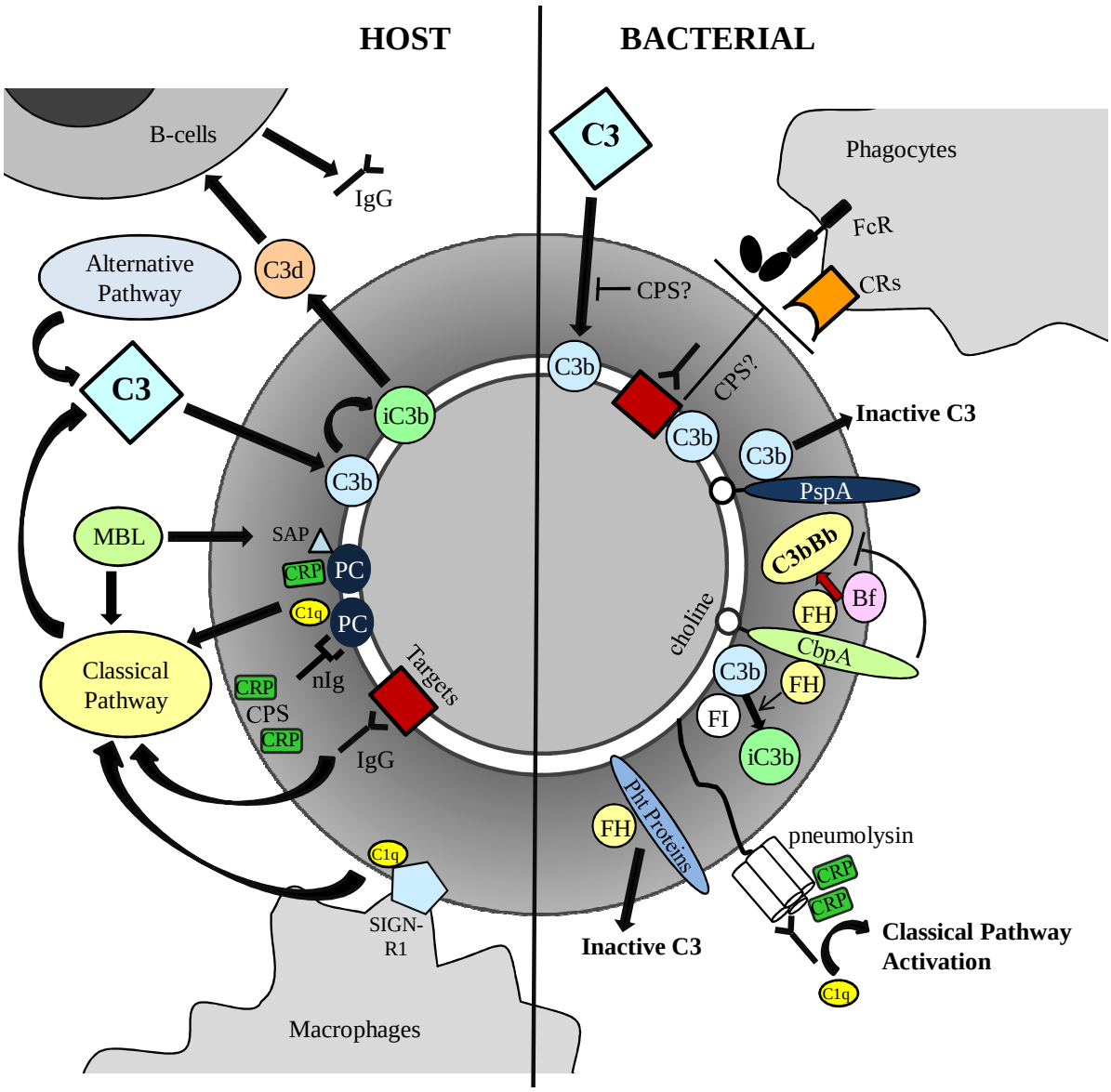


Fig 1.9 Interaction of *S. pneumoniae* with complement

Mechanisms of complement deposition on *S. pneumoniae* mediated by the host are shown on the left and *S. pneumoniae* mechanisms of resistance to complement deposition on the right. Yellow ovals represent C1q, blue triangle represent SAP and green rectangles CRP. Antibodies are shown as either specific IgG (triangular Fab) or natural Ig (rectangular Fab). The macrophage receptor SIGN-R1 (blue pentagon) is known to mediate phagocytosis of *S. pneumoniae* through the classical pathway. Pneumolysin was thought to be released into the serum and act to deplete complement, however needs amending given recent data indicating that it is cell wall attached.

1.5 THE POLYSACCHARIDE CAPSULE

S. pneumoniae expresses a polysaccharide capsule (CPS) which is required for full pathogenicity (Austrian 1981), and it has been demonstrated that unencapsulated mutants are highly attenuated in infection models (MacLeod et al. 1950; Brown et al. 1982; Watson et al. 1990; Morona et al. 2004). The polysaccharide capsule forms a highly hydrated 100-400nm thick shell, and is normally partially covalently attached to the bacterial cell wall. There are currently 91 known capsular serotypes, with the structure of the polysaccharides varying between the different capsular serotypes (Park et al. 2007). Most are complex structures that contain multiple sugars, linkages and frequently side chains, although the serotype 3 and 37 are composed of only one or two sugars and are relatively simple in structure.

1.5.1 Capsule Structure

Capsule polysaccharides are high molecular mass structures composed of different monosaccharides occurring in various combinations. The primary structure of the polysaccharides depends upon the nature and number of constituent monosaccharides, sequence and ring size of the monosaccharides, type and configuration of the glycosidic linkages and nonsugar substituents, and repeat an unknown number of times to produce the CPS (Jiang et al. 2001). The capsule polysaccharides are generally anionic due to the presence of uronic acid, phosphate and pyruvate, with the exception of the capsular polysaccharides of serotypes 7F, 7A, 14, 33F and 37 which have no net charge (Kamerling et al. 2000). In addition, the polysaccharide from serotype 1 has been found to have a zwitterionic charge, having an equal number of positively and negatively charged groups.

Some commonly occurring monosaccharides found in the capsule polysaccharides include α/β-D-glucose, α/β-D-galactose, α/β-L-rhamnose and N-acetyl-α/β-D-glucosamine (Kamerling et al. 2000). In general, monosaccharides are found as pyranose rings (a six-membered ring consisting of 5 carbon atoms and an oxygen atom), excluding D-galactose which can be found in capsule polysaccharides in both pyranose and furanose rings (consisting of 1 carbon and an oxygen atom), and D-ribose which only occurs as a furanose ring. Phosphate groups are common in capsular polysaccharides, and several capsular serotypes contain phosphodiester bridges between an aldose derivative and a monosaccharide, including serotype 6A and 6B. However phosphate groups can also be found as a backbone substituent. Some serotypes have only small differences in structure between them, with differences occurring in the position of a glycosidic linkage or a single monosaccharide (Kamerling et al. 2000). For example, the polysaccharide structures of serotype 6A and 6B differ only a single glycosidic linkage: in serotype 6A, the α-L-Rha residue is linked to the C-3 of the D-ribitol residue, whereas in 6B it is linked to the C-4 of the D-ribitol residue (Larm et al. 1976). Furthermore, the serotype 7A and 7F capsular polysaccharides have been found to contain a similar arrangement of monosaccharides, and the structural difference between these two polysaccharides is comprised of the presence or absence of a β-D-galactose terminal side-chain (Moreau et al. 1988; Backman-Marklund et al. 1990) (Fig 1.10 and 1.11).

Serotype	**Primary Structure of CPS**
1	→3)-AAT-α-D-Gal*p*-(1→4)-α-D-Gal*p*A-(1→3)-α-D-Gal*p*A-(1→
2	→4)-β-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→ 2 ↑ 1 α-D-Glc*p*A-(1→6)-α-D-Glc*p*
3	→3)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→
4	→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc-(1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(*S*)Pyr-(1→
6A	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→3)-D-Rib-ol-(5→*P*→
6B	→2)-α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→*P*→
7F	→6)-α-D-Gal*p*-(1→3)-β-L-Rha*p*2Ac-(1→4)-β-D-Glc*p*-(1→3)-β-D-Gal*p*NAc-(1→ 2 4 ↑ ↑ 1 1 β-D-Gal*p* α-D-Gal*p*NAc-(1→2)-α-L-Rha*p*

Fig 1.10 Primary Structure of repeating units of serotypes 1, 2, 3, 4 6A, 6B and 7F CPS

Gal, galactose; Glc, glucose; GalA, galacturonic acid; GalNAc, *N*-acetylgalctosamine; Rha, rhamnose; ManNAc, *N*-acetylmannosamine; FucNAc, *N*-acetylfucosamine; Ac, acetate; Rib, ribose; *P*, phosphate; *p*, pyranose Adapted from Kamerling in *Streptococcus pneumoniae*, Tomasz 2000

Serotype **Primary Structure of CPS**

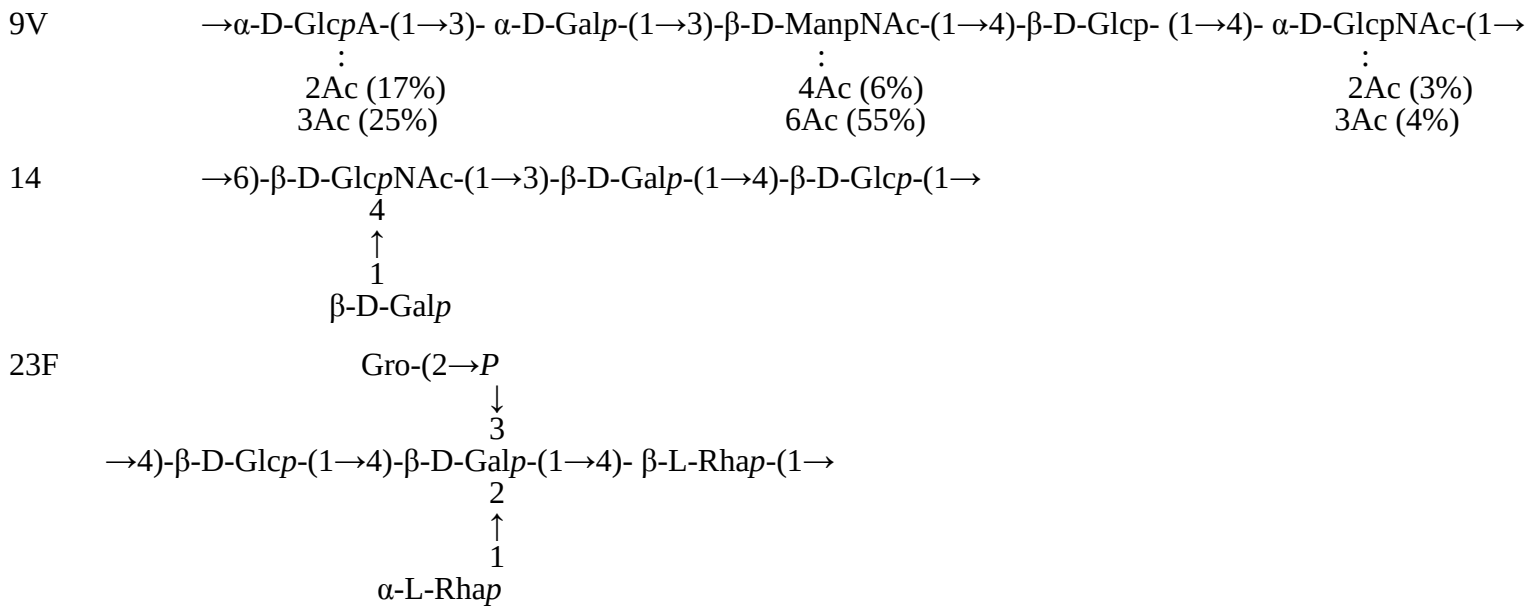


Fig 1.11 Primary Structure of repeating units of serotypes 9V, 14 and 23F CPS

Gal, galactose; Glc, glucose; Gro, glycerol; GlcNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalctosamine; Rha, rhamnose; ManNAc, *N*-acetylmannosamine; Ac, acetate; Rib, ribose; *P*, phosphate; *p*, pyranose. Dots indicate where alternative side chains may occur in serotype 9V. Adapted from Kamerling in *Streptococcus pneumoniae*, Tomasz 2000

1.5.2 Capsular Genetics

The pneumococcal capsule is encoded by a single genetic locus (*cps*) which has a cassette-like organization and appears to be organized as a single transcriptional unit. The type-specific biosynthetic genes are flanked by genes *dexB* and *aliA* which are highly homologous amongst different capsular serotypes, (Garcia et al. 1997; Bentley et al. 2006) and do not play a role in capsule synthesis. 7 of the 18 capsule polysaccharide component monosaccharides are available to *S. pneumoniae* through housekeeping metabolic pathways and the remaining 11 are encoded by genes in the *cps* locus, which is composed of type-specific genes involved in the biosynthesis of specific polysaccharides (Bentley et al. 2006). In addition to encoding for monosaccharides, the *cps* locus also encodes for polysaccharide polymerases, flippases and transferases (Yother 2004). Enzymes specific for the synthesis and polymerisation of CPS are found in the central region of the *cps* locus. Only one set of type-specific genes is present in any given strain of *S. pneumoniae,* and there is little homology between type-specific genes from different capsular serotypes (Fig 1.12). Only a few genes (*cpsA, cpsB, cpsC, cpsD,* and in most cases *cpsE,*) located at the 5' end of the *cps* locus are conserved between clusters, and these are involved in the processing, regulation and export of CPS and may be involved in attachment of polysaccharide to the bacterial cell wall (Fig 1.12) (Yother 2004; Bentley et al. 2006). Only *cpsA* is more than 90% identical in all gene clusters (Garcia et al. 2000). A functional promoter sequence (*cpsp*) has been located 30 nucleotides upstream of the first codon in the *cpsA* gene and the transcriptional start point of the *cps* operon has also been identified (Munoz et al. 1997).

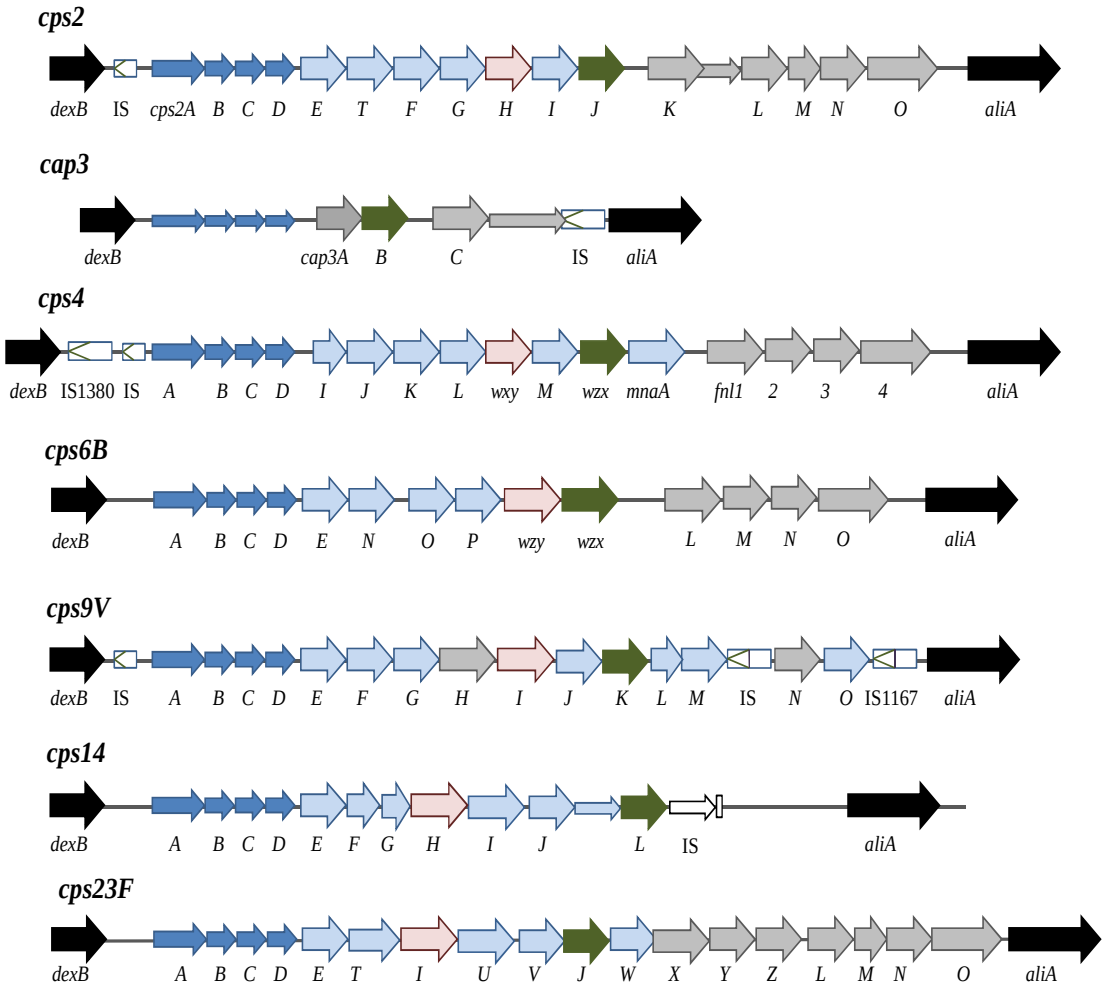


Fig 1.12 Organization of the *cps* loci from selected *S. pneumoniae* serotypes

ORFs within the DNA sequence are indicated by large boxed arrows showing common genes (dark blue), transferase genes (light blue), polysaccharide polymerase genes (pink), the synthase (glycosyltransferase) gene (green), activated sugar biosynthesis genes (grey) and IS sequences (white). Narrow boxes represent ORFs which are not required for CPS biosynthesis. Organisations based on published data for serotypes 2 (Iannelli et al. 1999), 3 (Arrecubieta et al. 1996), 4 (Jiang et al. 2001), 6B (Jiang et al. 2001), 9V (van Selm et al. 2002), 14 (Kolkman et al. 1997) and 23F (Morona et al. 1999). Figure adapted from Paton et al, 2005 and Yother, 2004.

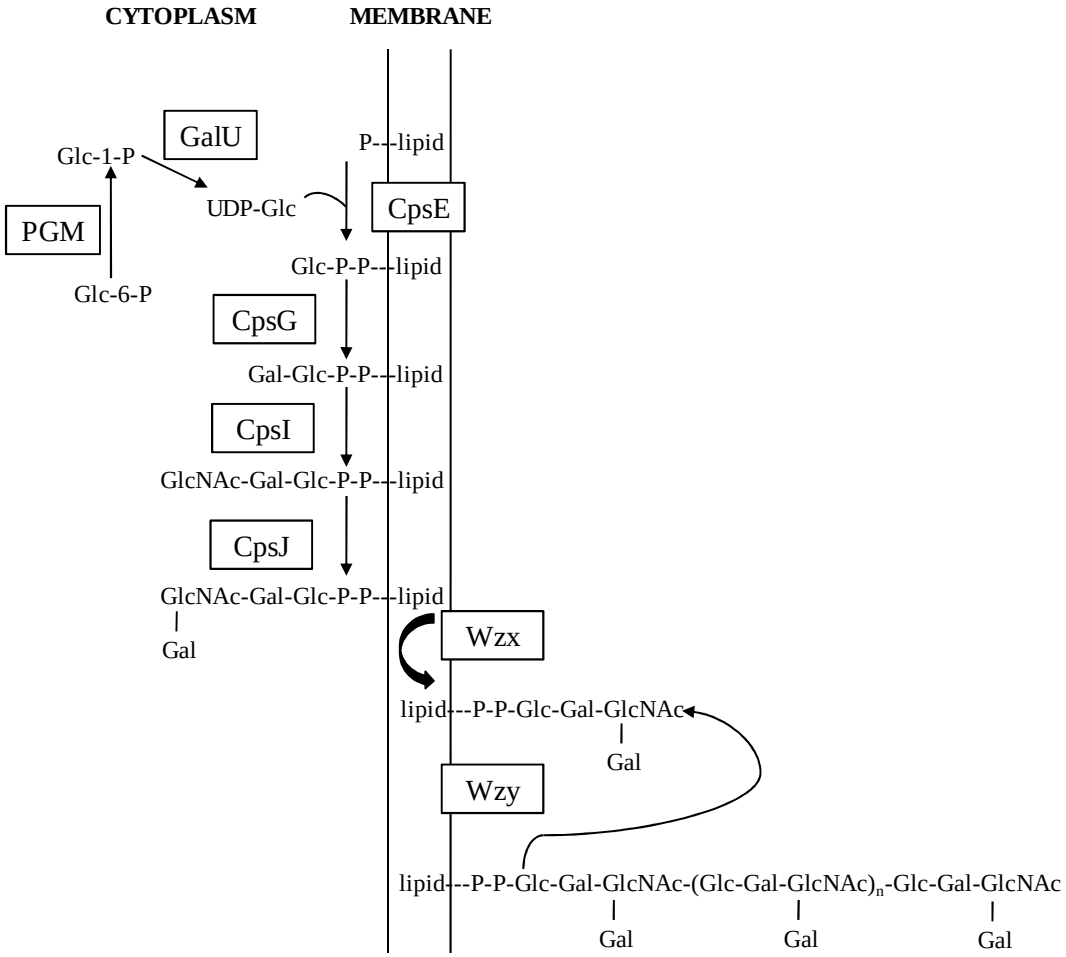
There are two genes which are not located within the *cps* locus are likely to be essential for formation of the CPS in all *S. pneumoniae* strains. Both PGM and GalU are required for the synthesis of UDP-glucose, which is a precursor for the biosynthesis of all pneumococcal CPS. A phosphoglucomutase (PGM) encoded by the *pgm* gene catalyses the conversion of Glu-1-P to Glu-6-P (Hardy et al. 2001). The other gene (*galU*) encodes a uridine diphosphate glucose pyrophosphorylase which catalyses the reversible formation of UDP-Glu from uridine 3-phosphate and Glu-1-P (Frey 1996). UDP-Glu is required for the synthesis of UDP-glucuronic acid and is also required for the interconversion of galactose and glucose. *S. pneumoniae* mutants with disruptions to either the *galU* or *pgm* gene produced no CPS and also exhibited growth defects (Mollerach et al. 1998; Cieslewicz et al. 2001).

1.5.3 Capsular Polysaccharide Biosynthesis

The biosynthesis of CPS is complex, with constituent monosaccharides being synthesised or taken up and converted to a nucleotide derivative before being linked on a membrane-bound lipid carrier (likely to be C_{55} undecaprenyl) (Cartee et al. 2005). In general pneumococcal CPS are synthesized by the Wzx/Wzy-dependent pathway, the genes for which are located invariably at the 5' end of the *cps* locus (*cpsA*, *cpsB*, *cpsC*, *cpsD*) (Bentley et al. 2006). The CPS is synthesized by transfer of an initial monosaccharide phosphate from a nucleotide diphosphate sugar to the undecaprenyl lipid carrier. Usually glucose-1-phosphate (Glc-1-P) is transferred from UDP-glucose onto a lipid acceptor (Kolkman et al. 1998; Yother 2004). However when the capsule contains galactose and not glucose, the initial step involves transfer of galactose-1-P (Gal-1-P) from UDP-galactose onto the lipid carrier (Yother 2004). This is followed by a sequential transfer of additional monosaccharides to produce the lipid-linked repeat

unit. This process is catalysed by glycosyl-transferases which are encoded within the serotype-specific regions of the *cps* locus (Kolkman et al. 1997). The lipid linked repeat unit is then transferred to the outer face of the cytoplasmic membrane by 'flippases' which contain approximately 12 membrane spanning domains and are homologous to the Wzx repeat unit transporter. Polymerisation occurs through the action of Wzy polymerase homologues to form mature CPS, which is then attached to peptidoglycan which anchors the CPS to the cell wall (Sorensen et al. 1990; Bentley et al. 2006) (Fig 1.13). However, it is unclear which enzyme is responsible for the polysaccharide transfer to the cell and at which precise point it occurs, however this process appears to be independent of CPS size (Bender et al. 2003).

In both serotype 37 and 3, the common sequences located at the 5' end of all other *cps* loci are either not present (serotype 37) or are mutated and not transcribed (serotype 3) (Paton et al. 2007). Serotype 3 and 37 are synthesized via the synthase pathway, in which CPS synthesis is catalyzed by a single, membrane bound glycosyltransferase (synthase) (Arrecubieta et al. 1996). This synthase is a member of the glycosyltransferase family 2, and the enzymes in this family have binding sites for nucleotide sugars and CPS, which allows transport of the polymer across the cytoplasmic membrane during synthesis (Campbell et al. 1997). For the serotype 3 polysaccharide initiation begins on a glycerophosphate lipid acceptor (Cartee et al. 2001) and is followed by addition of Glc and GlcUA (Cartee et al. 2000). Since the synthase does not have transmembrane domains such as those found in the Wzx transporter, the polysaccharide ejection mechanism is thought to result from premature translocation in the absence of UDP-Glc and UDP-GlcUA which leads to the CPS binding site failing to recognize the polymer (Forsee et al. 2000).



<u>Fig 1.13</u> The Wzx/Wzy dependent pathway for biosynthesis of serotype 14 CPS

Functions for the glycosyltranferases CpsE, CpsG, CpsI and CpsJ have been experimentally determined in serotype 14. PGM and GalU are the cellular enzymes and are not capsule specific. PGM, GalU, CpsG, CpsI and CpsJ are located in the cytoplasm, whereas CpsJ, Wzx transporter homologues and Wzy polymerases are membrane associated. Following the transfer of the chain, the lipid P-P is hydrolysed to lipid-P and recycled to the cytoplasmic face of the membrane.

Adapted from Yother, in The Pneumococcus, 2004

1.5.4 Regulation of capsule polysaccharide production

The ability of *S. pneumoniae* to regulate production of CPS at the transcriptional, translational or post-translational level is likely to be important for *S. pneumoniae* survival in different host environments. However, currently no transcriptional control elements have been identified in association with the *cps* promoter (Munoz et al. 1997), although there is evidence to suggest that the *cps* locus level of expression differs between opaque and transparent phase variants (Weiser et al. 2001). Additionally, approximately fourfold higher levels of *cps* mRNA, are found in *S. pneumoniae* isolated from the blood of infected mice compared to bacteria grown *in vitro* (Ogunniyi et al. 2002).

The *cpsA*, *cpsB*, *cpsC*, *cpsD* common genes found at the 5' end of the *cps* locus encode proteins which are involved in the modulation of CPS production. Whilst the precise role of CpsA is not known, *cpsA* deletion mutants produce less CPS (Morona et al. 2004). Interestingly a *cpsA* homologue in group B streptococci does seem to function as a transcriptional activator (Cieslewicz et al. 2001). CpsB, CpsC and CpsD act in a phophoregulatory pathway that regulates CPS production and chain length. CpsB (a manganese dependent phophotyrosine-protein phosphatase) and CpsC (a membrane protein) function together to regulate capsule assembly, export and attachment to the cell wall by tyrosine phosphorylation of CpsD. CpsC, CpsD and ATP interact to promote biosynthesis of CPS. This is followed by autophoshorylation of CpsD which slows capsule polysaccharide synthesis through changes in protein interactions. The CPS polymer is then transferred to the putative polysaccharide cell-wall ligase and finally CpsB dephoshorylates CpsD allowing for further polysaccharide synthesis to be promoted (Morona et al. 2000; Morona et al. 2004; Kadioglu et al. 2008) (Fig 1.14).

Deletion of *cpsC* or *cpsD* results in only a low level of CPS which is composed of short-chain polymers (Morona et al. 2000; Bender et al. 2003). Deletion of *cpsB* leads to increased phosphorylation of CpsD and increased capsule production (Bender et al. 2003).

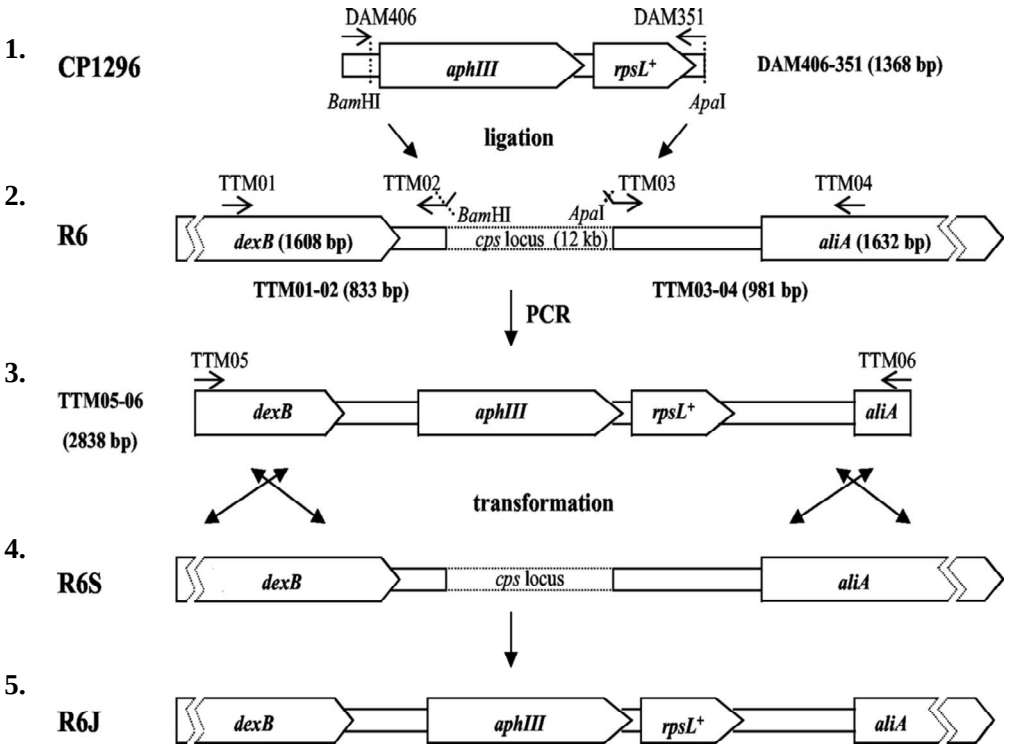
The transcription of the *cps* locus is also affected by RegM, a protein involved in the regulation of sugar-metabolism. This suggests that a carbon source could also affect capsular expression (Giammarinaro et al. 2002). Recently it was shown that serotypes producing polysaccharides that are less metabolically costly (ie with a low number of carbons and correspondingly low number of high-energy bonds or ATP-equivalents) tend to be more heavily encapsulated and therefore more likely to avoid phagocytic clearance and persist in carriage (Weinberger et al. 2009).

1.5.5 Capsular Switching

Capsular switching is clinically important in *S. pneumoniae* disease as this phenomenon may undermine the efficacy of current capsular polysaccharide conjugate vaccines. The introduction of the conjugate vaccine in children has caused a rapid and large decrease in the prevalence of vaccine serotypes in IPD and carriage. However, this has been associated with a rise in the prevalence of non-vaccine serotypes in both IPD and carriage (Long 2005; Beall et al. 2006; Munoz-Almagro et al. 2008). The cassette-like arrangement of the *cps* genes between genes which are highly conserved amongst all strains allows the naturally transformable *S. pneumoniae* to change serotype through recombinational exchange. Studies of antibiotic-resistant isolates show that serotype switching between different capsular serotypes occurs naturally within populations of

S. pneumoniae (McGee et al. 2001; Sandgren et al. 2004; Hanage et al. 2005; Brueggemann et al. 2007). The major multiresistant Spanish serotype 23F clone has undergone capsular switching on at least 4 occasions, transforming into serotype 19F (Coffey et al. 1998). Serotype 19A variants of the same multiresistant serotype 23F have also emerged through recombinational exchanges at the *cps* locus on more than one occasion (Coffey et al. 1998). Recombinations between serotype 9V and 14 loci can occur upstream of *dexB* and downstream of *aliA* or within the conserved TDP-Rha synthesis genes (Coffey et al. 1998).

Furthermore, capsular switching can be utilized to engineer strains of *S. pneumoniae* which are genetically isogenic except at the *cps* locus. The use of a Janus cassette has increased the identification of extremely low-frequency transformants, enabling this technique to be used to create multiple strains with the same genetic background but expressing different *cps* loci (Trzcinski et al. 2003). Furthermore there seems to be no detectable cost of this particular transformation of a strain and by the performance of backcross steps ensures a low likelihood of recombination replacements involving exogenous DNA taking place outside the *dexB-cps-aliA* locus (Fig 1.14) (Trzcinski et al. 2003). These strains are potentially a powerful experimental tool, since differences between these mutants must be due to the either the type or amount of CPS which is expressed. This enables studies on the effect of capsular serotype without the confounding of non-capsular genetic variation and without mutant strains suffering metabolic costs of the transformation.



<u>Fig 1.14</u> Construction of the Janus cassette in the R96 *S. pneumoniae cps* locus

dexB and *aliA* genes of R6 and *aphIII* (Km^r, Kanamycin resistant) and *rpsL*$^+$ (Sm^s, Streptomycin sensitive) genes of Janus (size of the ORF is in parentheses) are represented by pentagons. Oligonucleotides used to amplify cassette elements capitalised. Restriction sites of enzymes creating sticky ends before ligation steps are shown by dotted lines. The 2 top lines show elements of CP1296 and R6 used to construct the *dexB*-Janus-*aliA* cassette. The ligation product was used as a template to amplify the TTM05-06 fragment (3) and this was then used to transform R6S (4) to create R6J (5) using selection for resistance to kanamycin. Sizes of PCR products generated are given in brackets next to the product names. Adapted from Trizcinski et al, 2003.

1.5.6 Phase variation

S. pneumoniae undergoes spontaneous phase variation between two distinct colony forms, which are distinguishable based on colony morphology when viewed using oblique, transmitted light. Rates of phase variation between opaque and transparent phenotypes vary between 10^{-3} to 10^{-6} per generation depending on the isolate (Weiser et al. 1994). Transparent phenotypes are more adherent to epithelial cells and type II lung cells *in vitro* (Cundell et al. 1995), and are more efficient at nasopharyngeal colonization in animal models of carriage (Weiser et al. 1994). Opaque colonies appear dome-shaped, undergo spontaneous lysis less rapidly and are more frequently isolated from IPD (Serrano et al. 2006). Depending on the strain, opaque phase variants express a 1.2 to 5.6 fold greater amount of CPS compared to the transparent variant (Kim et al. 1998). However, outside of CPS expression there are also differences in other strain characteristics and structures which are summarized in Table 1.1.

Table 1.1 Summary of the characteristics and structure associated with the phase variation of *S. pneumoniae* Taken from Weiser 1998

Characteristic/Structure	Opaque Phenotype	Transparent Phenotype
Autolysis	↓	↑
LytA Expression	↓	↑
PspA Expression	↑	↓
CbpA Expression	↓	↑
Teichoic Acid Content	↓	↑
Nasopharyngeal colonization (infant rat)	↓	↑
IP virulence (adult mice)	↑	↓

Phenotypic variation also occurs in GBS, in which populations coexist with different capsule thicknesses, allowing for phase shifting towards a less encapsulated form which adheres well to epithelial cells or a phase of abundant encapsulation which adheres poorly (Rubens et al. 1987; Philips et al. 1992; Cieslewicz et al. 2005). *N. meningitidis* also varies the level of CPS with changes in pH and nutrient-limiting conditions, however these changes are reversible and due to changes in the number of cytidine residues within the 5' region of the α-2,8-polysiayltransferase gene, an enzyme found on the inner membrane which is responsible for capsule formation (Hammerschmidt et al. 1996). In *S. pneumoniae* a genetic locus able to transform a transparent strain recipient to an opaque phenotype identified two genes, *glpD* and *glpF* which may be involved in regulation of phase variation although the precise mechanism remains unclear (Weiser 1998). Both these genes have homology to the glycerol regulon genes in other bacteria and are followed by a sequence with homology to other repetitive pneumococcal intergenic elements (BOX A and C) upstream of an ORF which may encode a 126 amino acid protein. There is also a second longer ORF which is transcribed in the opposite orientation, and neither ORF has any significant homology to current entries in sequence databases (Weiser 1998). Interestingly this second ORF has an associated area of 19 tandem thymidine residues located upstream, and these have been shown to create 'molecular switches' associated with phase variation (Weiser et al. 1989). Hence a stem-loop forming element which is known as BOX A-C has been implicated in the control of opaque phase variation. However the stimuli and signals which stimulate phase switching and the exact mechanism by which this occurs remains poorly understood.

1.5.7 Role of polysaccharide capsules in other species

The presence of a polysaccharide capsule surrounding the cell wall is not unique to *S. pneumoniae*, and many pathogenic Gram positive and negative bacteria species possess a capsule. Bacterial surfaces contain various structures which induce host immune responses, and in general bacterial capsules that completely surround bacteria mask underlying cell surface structures which would otherwise be potent activators of the complement system. Generally the capsules which surround bacteria have surfaces which are poor activators of complement, thereby masking potent complement activators and replacing the bacterial cell surface with a poor target for complement. For example, the K1, K10 and K16 capsules of *Klebsiella pneumoniae* have been shown to prevent the complement system activating by masking surface structures (Salo et al. 1995), with the further consequence of reduced opsonophagocytosis. Certain bacterial capsules, such as the K66 *K. pneumoniae* strain, are co-expressed with strong complement activating structures of the bacterial outer membrane (Tomas et al. 1991). In this particular strain, the capsule masks the C3b (which is deposited on the O antigen LPS chain) from phagocytes, thereby reducing opsonophagocytosis.

The *K. pneumoniae* serotype K1 capsule has been shown to have an effect against antibody, and acts as a barrier against O-specific antibodies (Cryz et al. 1986). The serotype K2 polysaccharide capsule acts as a partial barrier, and this effect of the *K. pneumoniae* capsule against antibody seems to be serotype specific (Meno et al. 1990; Held et al. 2000). In addition, masking by capsular polysaccharides may induce a weak or absent immune response to the bacteria, which may even induce tolerance to foreign antigens in the host. GBS that produce higher levels of type-III polysaccharide are more virulent that strains which produce lower levels (Rubens et al. 1987; Philips et al. 1992),

and this polysaccharide has been shown to mask cell wall complement-activating structures (Hulse et al. 1993; Tamura et al. 1994) and prevent C3 deposition (Marques et al. 1992). In addition several Gram positive bacteria prevent the antibody activation of complement (Corbeil 2000). IgA antibody directed against the group C *N. meningitidis* capsule also acts to prevent IgG mediated complement killing (Jarvis et al. 1991).

The sialic acid found in the *N. meningitidis* capsule acts to inhibit the alternative complement pathway by binding factor H, which mimics the effect of sialylation on cell membranes in reducing the amount of C3b/iC3b deposited (Ram et al. 1998). Furthermore it is thought that sialylation of the group B streptococcus polysaccharide capsule increases FH binding and thereby reduces C3 deposition (Wessels et al. 1989; Vimr et al. 2002; Lewis et al. 2004). Sialylation of the LPS in non-typable *Haemophilus influenzae* also inhibits C3 deposition although this does require FH binding (Figueira et al. 2007).

Polysaccharide capsules may also prevent the activation of terminal complement MAC in Gram negative bacteria (Gram positive bacteria are resistant to MAC lysis due to the presence of a rigid and thick peptidoglycan layer). In *K. pneumoniae* and *Serratia marcescens* this is thought to occur by preventing MAC binding or forcing the MAC to bind too far from the bacterial cell surface to be effective (Alberti et al. 1996). Additionally in these strains the MAC associates to O-antigen side chains on LPS by weak ionic interactions, so that it is shed and therefore not effective in lysing the bacterium.

Extracellular pathogens possess polysaccharide capsules which inhibit phagocytosis by preventing phagocyte recognition and/or ingestion of bacteria. For example, the N-acetylneuraminic acid capsule of *Neisseria meningitidis* and K1 *E. coli* strains surrounds the bacteria, weakly activates complement providing a poorly opsonised external surface in the absence of specific antibodies and make ligands inaccessible to phagocytes (Kim et al. 1992; Read et al. 1996; Virji et al. 1996). Furthermore some airway pathogens possess capsules which may down-regulate IL-6 and IL-8 cytokine expression which decreases plasma cell maturation and thereby amount of antibody, hence slowing the migration of phagocytes towards infection sites. This has been demonstrated in *Actinobacillus actinomycetemcomitans* serotype b and some *K. pneumoniae* serotypes (Yoshida et al. 2001; Ohguchi et al. 2003).

Polysaccharide capsules are also present on some fungi, and *Cryptococcus neoformans* is a haploid yeast which is surrounded by a capsule composed primarily of glucuronoxylomannan (Cherniak et al. 1980). The *C. neoformans* capsule is essential for cryptococcosis in mice and unencapsulated strains are easily phagocytosed by both neutrophils and macrophages (Bulmer et al. 1967; Bulmer et al. 1968; Kozel 1977). Inhibition of phagocytosis is mediated directly by glucuronoxylomannan component of the capsule, which also enables adherence to type II alveolar epithelial cells via the CD14 receptor (Kozel et al. 1976; Barbosa et al. 2007). Furthermore the glucuronoxylomannan interferes with neutrophil migration, suppresses T cell immunity and up-regulates cytokine production (Murphy et al. 1982; Retini et al. 1996; Lipovsky et al. 2000; Ellerbroek et al. 2004). In contrast to other polysaccharide capsules, the cryptococcal capsule is a potent activator of the complement system, with encapsulated yeast cells binding 10^7-10^8 C3 fragments, mostly in iC3b form (Young et al. 1993).

Complement activation occurs solely through the alternative pathway which produces an asynchronous focal initiation because of random deposition of fluid-phase C3b onto the capsule, which is followed by amplification leading to complete capsule coverage with C3 (Kozel et al. 1991). This acts as a diversion technique for avoiding complement deposition on the yeast cell surface.

In summary, the capsules of other microbial pathogens have been shown to have a variety of effects on complement and phagocytosis that will aid immune evasion.

1.5.8 Role of the *S. pneumoniae* capsule

The capsule has been shown to be vital for virulence of *S. pneumoniae*, and unencapsulated strains are not recoverable from animal models of disease. Furthermore transparent strains, which express a thinner polysaccharide capsule, show enhanced colonization compared to opaque strains, and capsule is required for sustained colonization of the nasopharynx, though strains with less polysaccharide retain the ability to colonize (Weiser et al. 1994; Kim et al. 1998; Magee et al. 2001). Recent studies have utilized unencapsulated mutants transformed from virulent strains of *S. pneumoniae* to confirm that the capsule is required for virulence (Hardy et al. 2001; Magee et al. 2001). Furthermore opaque phase variants are more virulent in systemic disease than their transparent phase counterparts (Kim et al. 1998). It has previously been demonstrated that different *S. pneumoniae* capsular serotypes vary in their ability to cause invasive disease (Hausdorff et al. 2000), and this may be due to differences in the chemical structure of the capsular polysaccharides of which the capsule is comprised. Interestingly despite differences in the range serotypes which cause animal

models of pneumococcal disease, there is still a limit to the number of serotypes which do so. Furthermore virulence of *S. pneumoniae* in these models correlates with serotype (Briles et al. 1992; Wu et al. 1997).

The capsule is believed to protect the bacteria through its effect against complement and phagocytosis. This is thought to occur by blocking access to C3b which is localized on the cell wall beneath the polysaccharide capsule, with studies showing alternative pathway activation by the *S. pneumoniae* wall leading to increased deposition of C3b on the cell wall (Abeyta et al. 2003). However earlier studies found similar levels of C3b deposited on the cell wall fragments obtained from encapsulated and unencapsulated through activation of the alternative complement pathway (Winkelstein et al. 1976; Winkelstein et al. 1980; Brown et al. 1983). Therefore the capsule may act to both limit C3 access as well as the amount of complement which is deposited on the bacteria. However CPS may also act as a target for complement mediators, providing a mechanism for complement mediated immunity. Recently a classical pathway fixation pathway was reported in which capsule polysaccharide is targeted by SIGN-R1 (Kang et al. 2006) further outlining the importance of the classical pathway in host immunity to *S. pneumoniae* (Brown et al. 2002). Furthermore both SAP and CRP may target the capsule polysaccharides as well as phosphocholine on cell membranes, DNA and chromatin (Volanakis et al. 1971; Volanakis et al. 1979; Hind et al. 1984; Loveless et al. 1992; Li et al. 1994). Overall the precise role of the capsule in preventing complement deposition remains confused and unclear.

There is good evidence that capsular effects on immune function vary with serotype. Complement deposition and activation varies between clinical isolates of different

serotypes, and there are also differences in phagocytosis between serotypes (Wood et al. 1949; Fine 1975; Giebink et al. 1977; Winkelstein et al. 1977; Braconier et al. 1982; Gordon et al. 1986; Hostetter 1986; Melin et al. 2009). Abeyta and Yother demonstrated that the switching of a serotype 2 locus with a serotype 3 locus resulted in a level of C3 deposition on the mutant strain that was between that of the wild-type D39 and WU3 strains, which was reflected in differences in the levels of antibody binding (Abeyta et al. 2003). The capsular switching technique was used by Kelly and Yother, who converted strains of serotype 2, 5 and 6B to serotype 3. In a mixed result, replacement of the serotype 2 capsule did not affect virulence in a mouse model, whereas replacement of serotype 5 and 6B had profound effects on the virulence of the *S. pneumoniae* strain (Kelly et al. 1994). This indicates the capsule's key role in determining complement access to the cell surface of *S. pneumoniae*, however complement deposition seem to be affected by both capsular serotype and other non-capsular genetic variation. Non-capsular genetic variation may account for differences in expression and structure of numerous virulence factors, including CbpA, neuraminidase, autolysin, pneumococcal surface protein A (pspA), hyaluronidase and pneumococcal adhesins. The *S. pneumoniae* capsule surrounds many of these virulence factors and possibly affects interactions between these pneumococcal proteins and host immune mechanisms. Therefore variation in the structure of multiple virulence factors combined with differences in the effect of different capsule polysaccharides may account for differences in complement deposition between strains of different serotypes as well as between strains of the same capsular serotype.

Phagocytosis of *S. pneumoniae* plays an important role in clearing pneumococcus from the lungs during pneumonia, and the capsule is believed to inhibit phagocytosis (Wood

et al. 1946; MacLeod et al. 1947). Previous studies have shown that the polysaccharide capsule from several serotypes inhibits phagocytosis and that serotypes expressing thicker polysaccharide capsules are more resistant to phagocytosis (Wood et al. 1949; MacLeod et al. 1950). However these experiments were performed using strains which are now known to have significant non-capsular genetic variation or using dead bacteria (Wood et al. 1949; Lanie et al. 2007). More recent experiments have shown that unencapsulated mutants have are more susceptible to phagocytosis and there is limited data showing increased levels of complement deposition on their surface (Winkelstein 1981; Rubens et al. 1987; Abeyta et al. 2003; Quin et al. 2007). Complement is required for efficient opsonisation, and it remains unclear if the capsule's effect on phagocytosis is a down-stream consequence of its ability to inhibit complement deposition. Indeed recent data showing increased phagocytosis of an unopsonised unencapsulated serotype 6B strain suggests there can be a capsular effect on non-opsonic phagocytosis (Weinberger et al. 2009). Furthermore the capsule has an inhibitory effect on macrophage phagocytosis and an unencapsulated TIGR4 strain has been shown to induce increased apoptosis in monocyte derived macrophages (Jonsson et al. 1985; Ali et al. 2003). Recently is has been suggested that the structure of the polysaccharide predicts prevalence of a given serotype, with common carriage strains of *S. pneumoniae* showing increased capsule thickness and resistance to non-opsonic phagocytosis by neutrophils (Weinberger et al. 2009). This may be important as effects of capsule on phagocytosis are likely to reduce complement mediated uptake (Yuste et al. 2008). However, other data has shown that the capsule prevents *S. pneumoniae* killing by neutrophil extracellular traps and assists nasopharyngeal colonization through charge-dependent interactions with nasal mucous rather than inhibition of phagocytosis or complement-mediated immunity (Nelson et al. 2007; Wartha et al. 2007).

Despite the studies described above, the mechanisms by which the capsule prevents complement deposition on *S. pneumoniae*, which complement pathways are involved and the differences in the pattern of complement deposition on encapsulated and unencapsulated are poorly understood. Whether the effects of the capsule on *S. pneumoniae* phagocytosis are solely attributable to inhibition of bacterial opsonisation with complement or are also consequences of the effects of the capsule on other mechanisms of phagocytosis is also poorly defined. As there are few data on which effects of the capsule are relevant during invasive infection, the relative importance during systemic infection of capsule inhibition of complement-mediated immunity to *S. pneumoniae* compared to other effects of the capsule on interactions with the host remains unclear.

1.6 SUMMARY AND HYPOTHESIS

S. pneumoniae is the commonest cause of pneumonia in the UK and is also a common cause of other respiratory infections including bronchitis and empyema. A vital component of systemic immunity to *S. pneumoniae* is complement, and patients with complement deficiencies and animal models of infection using complement deficient mice show increased disease severity. The pneumococcal capsule is the main virulence factor of *S. pneumoniae* virulence however surprisingly little is known about the precise effect of the capsule on interactions with host immunity. Previous studies have provided conflicting data about the role of the capsule in preventing complement deposition on the bacterial cell surface. The *S. pneumoniae* capsule is thought to be anti-phagocytic and it is also unclear as to whether this is an inherent property of the polysaccharides or a downstream consequence of any anti-complement or antibody effect.

Hence the effect of the capsule on innate immune mechanisms remains undefined. In addition the effect of the *S. pneumoniae* capsule during early lung infection. AMs are the resident specialised phagocytic cell in the lung and an important modulator of inflammatory responses, and are likely to be the first immune cell encountered by *S. pneumoniae* in the lung. The capsule may affect interactions with AMs however this remains unclear from existing data. Furthermore it since complement levels in BAL fluid are about 10% of that found in serum it is unclear as to whether complement mediates an effective pulmonary immune mechanism and whether the capsule modulates this response in pulmonary disease.

As the capsule is the current target used in vaccination against *S. pneumoniae* it is important to clarify the effect of the capsule on host immunity. There is only limited evidence for the efficacy of conjugate vaccines against pneumococcal pneumonia and innate immune responses may have a significant effect on adaptive immune responses to vaccines. Hence any difference in the effect of capsule between different *S. pneumoniae* serotypes on innate immune mechanisms may affect the efficacy of the conjugate vaccine.

Different serotypes and clones of *S. pneumoniae* have differing abilities to cause invasive disease but as yet no mechanism has been described which fully accounts for this phenomenon. The ability of different capsular polysaccharides to affect complement deposition is unknown as previous studies showing differences in complement deposition between serotypes have been confounded by non-capsular genetic variation. In addition the relative contribution of variation in capsular serotype and other non-capsular pneumococcal structures to complement mediated immunity, phagocytosis and virulence remains unclear.

This thesis will therefore address the following hypothesis:

The *S. pneumoniae* capsule aids virulence by preventing complement dependent and independent interactions with phagocytes.

The specific aims of this thesis are to:

(i) Clarify the effect of the capsule on complement deposition on *S. pneumoniae* and

investigate potential mechanisms by which the capsule can reduce complement activity against *S. pneumoniae*.

(ii) Characterise the effects of the capsule on interactions with PMNs, and determine if these are complement dependent or independent.

(iii) Investigate the effects of the capsule on interactions with macrophages and the capsule's role in early lung infection

(iv) Determine if capsular serotype affects complement mediated immunity.

(v) Determine if non-capsular genetic variation affects complement mediated immunity.

CHAPTER 2

MATERIALS AND METHODS

2.1 BACTERIAL STRAINS

2.1.1 Bacteria

The parental (WT) strains of *Streptococcus pneumoniae* and their respective unencapsulated mutants that were used in these experiments are:

- TIGR4: a highly virulent capsular serotype 4 isolated from the blood of a 30 year old male patient in Kongsvinger, Norway. It was given as a gift from Prof J Weiser, University of Pennsylvania.
- TIGR4*cps*: TIGR4 strain containing a Janus cassette in place of the capsule gene locus, conferring resistance to Kanamycin (Trzcinski et al. 2003). Also given as a gift from Prof J Weiser, University of Pennsylvania.
- D39: An important, invasive serotype 2 strain that was used in experiments by Avery and co-workers to demonstrate that DNA is the genetic material (Paton et al. 1993).
- D39-DΔ: the D39 WT strain with an insertion duplication mutation using pVA891 in part of the capsule locus (cps 2D) (Paton et al. 1993).
- Serotype 3: Strain 0100993 was obtained from a human clinical pneumonia isolate, originally from SmithKline Beecham. This strain was given as a gift from Dr D Holden, Imperial College.

2.1.2 TIGR4 Capsular Switch Strains

S. pneumoniae TIGR4 genetic background strains which have been genetically modified at the capsule gene locus but which are otherwise isogenic, were given as a gift from

Prof J Weiser, University of Pennsylvania. The TIGR4 capsule genetic locus was replaced with a Janus cassette, and this cassette was then replaced with the capsule genetic locus from a different serotype by researchers in Prof Weiser's laboratory (Trzcinski et al. 2003). Furthermore these strains were separated into opaque and transparent phenotypes and checked regularly throughout the work of this thesis, and the Quelling Reaction performed to check capsular serotype. These strains are fully listed in Table 2.1.

2.1.3 Clinical Isolate Strains

Strains isolated from blood or cerebrospinal fluid (CSF) of children under the age of 5 in Finland in 2002 (Hanage et al. 2005) was given as gifts from Dr Hanage and Dr Spratt. These strains comprised of 5 different capsular serotypes, and within each serotype there were 4 different strains based on MLST, with some of the strains having a known invasive potential. These strains are listed in full in Table 2.2, and when phenotyped were all found to be in opaque phase.

The strains provided by Dr Henriques-Normark were isolated from patients in Sweden and are of different MLST backgrounds covering a range of serotypes, but all these strains were of a known invasive potential, and are listed in Table 2.3.

Table 2.1 TIGR4 and D39 background strains, provided by Prof Jeffrey Weiser, University of Pennsylvania, USA or Prof James Paton, University of Adelaide.

Name	Background Strain	Capsule Serotype	Phase Variation	Antibiotic Resistance
TIGR4	TIGR4	Serotype 4 (WT)	-	Streptomycin-R
TIGR4*cps*	TIGR4	Unencapsulated	-	Streptomycin-S Kanamycin-R
D39	D39	Serotype 2 (WT)	-	-
D39-DΔ	D39	Unencapsulated	-	Erythromycin-R
P1637	TIGR4	Serotype 6A	Opaque	Streptomycin-R
P1638	TIGR4	Serotype 6A	Transparent	Streptomycin-R
P1688	TIGR4	Serotype 7F	Opaque	Streptomycin-R
P1689	TIGR4	Serotype 7F	Transparent	Streptomycin-R
P1691	TIGR4	Serotype 23F	Opaque	Streptomycin-R
P1692	TIGR4	Serotype 23F	Transparent	Streptomycin-R
P1702	TIGR4	Serotype 4	Opaque	Streptomycin-R
P1701	TIGR4	Serotype 4	Transparent	Streptomycin-R

-R, Resistant

-S, Sensitive

Table 2.2 Clinical isolate strains taken from blood and CSF of children under 5 years old in Finland 2002 MLST performed by Dr William Hanage, Imperial College, London.

Serotype	Strain Type	Strain Name	Lab Name	Odds Ratio for Invasiveness
4	205	M127	M127	-
4	259	M313	M313	-
6A	518	IOKOR801-2	6Aa	-
6A	490	IOKOR1373-9	6Ab	-
6A	488	IOKOR1277-3	6Ac	0.48
6A	1068	IO13048	6Ad	-
6B	138	M7-6B	6Ba	2.45
6B	273	JJ270-6B	6Bb	-
6B	90	M225-6B	6Bc	-
6B	176	M49-6B	6Bd	0.97
14	124	M117-14	14a	2.57
14	162	M65-14	14b	1.37
14	156	M134-14	14c	10.1
14	307	PJ581/14	14d	-
23F	36	OXC-1417-23F	23Fa	0.67
23F	515	IOKOR706-5	23Fb	-
23F	37	IOPR1592	23Fc	0.48
23F	277	JJ279-23	23Fd	-

Table 2.3 Clinical isolate *S. pneumoniae* strains given by Dr Birgitta Henriques-Normark. MLST performed in Karolinska Institute, Sweden.

Serotype	Strain Type (ST)	BHN Number	Odds Ratio for Invasiveness
1	306	BHN 30	9.6
1	228	BHN 32	9.6
1	217	BHN 166	9.6
4	1222	BHN 42	12.1
4	205	BHN 43	12.1
6B	138	BHN 49	0.6
6B	176	BHN 50	0.6
9V	162	BHN 62	1.5
9V	162	BHN 63	1.5
9V	156	BHN 69	1.5
14	124	BHN 84	4.4
19F	425	BHN 97	0.6
19F	162	BHN 100	0.6
19F	236	BHN 388	0.6
19F	556	BHN 95	0.6

2.2 MEDIA AND GROWTH CONDITIONS

2.2.1 Bacterial Culture

Bacteria were cultured at 37 °C in 5% CO_2 on 5% blood Columbia agar (Oxoid) plates, made using defibrinated horse blood (TCS Biosciences). Working stocks were made by transferring one colony of *S. pneumoniae* to Todd-Hewitt broth (Oxoid) with 0.5% yeast extract (Oxoid) (THY) and grown to an optical density (OD) between 0.3 and 0.4 (corresponding to a colony forming unit count of 10^8 CFU/ml). 10% glycerol was added and bacteria were stored in single use aliquots at -80 °C. The exact numbers of bacterial cell per ml was determined by plating tenfold serial dilutions of an aliquot onto Columbia blood agar, culturing overnight and counting the number of colonies to give a value for the colony forming units (CFU) per ml.

2.2.2 Synthetic Medium

C_{den} medium was used for growth of *S. pneumoniae* in conditions which required synthetic medium (Tomasz et al. 1964) (a full recipe is listed in Appendix I).

2.2.3 Growth Curves

2 x 10^7 CFU *S. pneumoniae* were inoculated into 6mls of THY Broth and incubated at 37°C for 2 hours. Under sterile conditions, 1ml of culture broth was transferred into a sterile cuvette in triplicate for each strain tested. The OD was then measured in a spectrophotometer at 580nm every hour to produce a growth curve in medium.

For growth in blood or serum, 50mls of blood was taken from a human volunteer and 15ml was heparinised and the remaining 35mls used to produce serum. 2 x 10^6 CFU *S. pneumoniae* were inoculated into 1ml of blood or serum and then incubated at 37°C for

4, 6 or 8 hours. At each time point, serial dilutions were made and plated onto blood agar plates which were incubated overnight at 37°C to allow a calculation of the CFU bacteria per ml of blood or serum for each strain tested.

2.2.4 Opaque and Transparent Phenotyping

Broth cultures of *S. pneumoniae* were streaked onto Tryptone Soy (Oxoid) plates onto which 5000 units of bovine catalase (Sigma) had been spread. Cultures were grown overnight in a 5% CO_2 incubator, after which colony morphology was assessed under transmitted illumination and magnification as previously described (Weiser et al. 2001).

2.2.5 FAM-SE Labelling

S. pneumoniae was cultured overnight on 5% blood Columbia agar at 37°C in 5% CO_2. A single loop of this culture was inoculated into 0.5% THY and grown to an OD between 0.7 and 0.8 (a mid log phase OD_{600}). 15ml bacteria were harvested by centrifugation and washed once with 5ml 0.1M sterile $NaHCO_3$, resuspended in 1ml bicarbonate buffer and 50µl FAM-SE solution (Cambridge Bioscience) (10mg/ml in DMSO) and incubated for 1 hour at 37 °C in 5% CO_2 without shaking. The cells were then washed 6 times in opsonophagocytosis buffer (Hanks balanced salt solution (HBSS) (GIBCO) o with 0.2% bovine serum albumin), until no free dye could be seen in the supernatant. Aliquots of FAM-SE labelled cells were made, 10% glycerol was added and the bacteria stored at -80 °C, protected from light.

2.3 SERUM AND ANIMALS

2.3.1 Serum Collection

Human serum was obtained from healthy volunteers, pooled and stored at -70 ℃ as single use aliquots. Before use human serum was thawed on ice and diluted using PBS [137mM NaCl, 2.7mM KCL, 8.1mM Na_2HPO_4, 1.5mMKH_2PO_4, pH 7.2-7.4] to the required concentration. Mouse blood was obtained by terminal cardiac puncture, clotted to produce serum and stored in the same manner as human serum. Furthermore, commercially obtained serum was purchased which had been depleted of either C1q, factor B, C9 or C3 (Calbiochem). This serum was also thawed on ice, divided into single use aliquots and stored at -70°C.

2.3.2 Complement Inactivated Serum

Serum was treated at 65°C for 20 minutes to inactivate complement but retain antibody activity. The complement activity of the serum was measured using the method listed below in 2.3.3 and the antibody activity confirmed by ELISA for capsular specific IgG and IgM (data not shown).

2.3.3 Complement Activity

The activity of the classical and alternative complement pathways sera was assessed using complement activity kits (The Binding Site). Briefly, following the manufacturer's instructions, calibrators and controls were resuspended, diluted and 5µl of each added to appropriate wells along with samples. The plate was left overnight at 4°C and then incubated at 37°C for 2 hours. The lysis zone around each well was measured using a x10 magnifier with graticule (Agar Scientific) and activity of each

sample was calculated from the resulting calibration curve (see Fig 2.1) (Yuste et al. 2008).

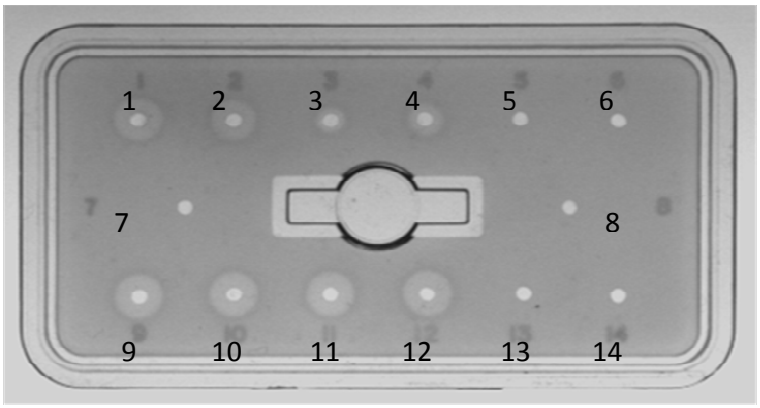
2.3.4 Capsule Serotype Specific Antibody Measurement

IgG and IgM levels were ascertained in serum using the WHO protocol (Wernette et al. 2003). 96 well flat-bottomed plates were plated overnight with dilutions of capsule polysaccharide extracts and washed with TBS [20mM Tris, 150mM NaCl, pH 7.6] Tween 0.1%. 89-SF serum was used as a reference serum and diluted in buffer with cell wall polysaccharide (CWPS) and serum samples were diluted in buffer with CWPS and 22F polysaccharide (to avoid non-specific binding). Following incubation for 30 minutes at room temperature, 50µl serum dilutions were added to each well and followed by a 2 hour incubation at room temperature. Plates were washed with TBS Tween 0.1% and 100µl 1:20,000 anti-human IgG or IgM (Sigma) was added per well. This was allowed to incubate for 2 hours at room temperature, plates were washed again and then 100µl 1mg/ml p-Nitrophenyl Phosphate (pNNP) in 1M DEA/0.5mM $MgCl_2$ was added to each well. Plates were transferred to the dark and the substrate allowed to develop until approximately OD 1.0. Plates were read using a reader at 450nm and 620nm signals and the final optical density calculated by subtracting the blank well readings from each signal and then subtracting the 450nm reading from the 620nm reading.

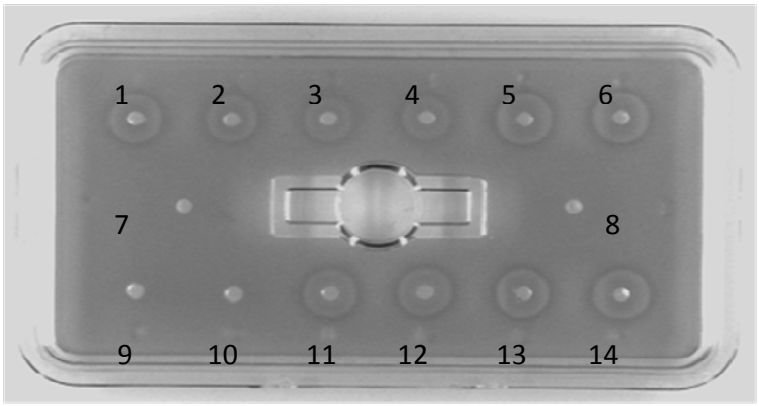
2.3.5 Serum IgG depletion using IdeS

IdeS (Immunoglobulin G-degrading enzyme of *Streptococcus pyogenes*) is a cysteine proteinase which cleaves IgG with a unique degree of specificity in the hinge region (von Pawel 2002, Wenig 2004). Purified IdeS was given as a kind gift from Dr Lars Björck. 1% IdeS or BSA (as a negative control) was incubated with 25% human serum

A



B



C

Serum	Alternative Pathway Activity	Classical Pathway Activity
Normal	-	100%
Heat Treated	0%	0%
C1q Depleted	85%	0%
C1q Depleted + C1q	86%	100%
Factor B Depleted	0%	100%

Fig 2.1 Complement activity of serum used in this thesis
(A) Alternative complement activity in control wells (1-4), heat treated serum (5, 6), C1q depleted serum (9, 10), C1q depleted serum with C1q protein added (11, 12) and Factor B depleted serum (13, 14). **(B)** Classical pathway complement activity in control wells (1-4), normal serum (5, 6) heat treated serum (7, 8), C1q depleted serum (9, 10), C1q depleted serum with C1q protein added (11, 12) and Factor B depleted serum (13, 14). **(C).** Final percentage complement pathway activity of different sera tested.

for 45 minutes at 37°C to cleave the heavy chains of IgG. This serum was then used in complement and IgG binding assays as well as opsonophagocytosis assays.

2.3.6 Animals

CD1 mice were purchased from Charles River and used in pneumonia model experiments. WT, *C1qa*$^{-/-}$, *Bf*$^{-/-}$ and C3$^{+/-}$ C57BL/6 mice were obtained from Marina Botto (Imperial College). All mice were sex matched in experiments and used at 6 weeks of age.

2.4 ANTIBODIES

Antibodies used in the work presented in this thesis were used under optimised conditions for temperature, duration of incubation and antibody dilution. A variety of primary antibodies directly conjugated were utilised, as well as unconjugated primary antibodies which required a conjugated secondary antibody. A full list of antibodies used for this thesis is detailed in table 2.4 and 2.5.

Table 2.4 Antibodies used in the complement factor work in this thesis

Target	Monoclonal/ Polyclonal	Host Species	Conjugation	Company	Dilution	Incubation Duration
Human Complement C3	Polyclonal	Goat	FITC	Cappel	1:300	30 mins
Human Complement C3	Polyclonal	Goat	HRP	MP Biomed	1:4000	1 hour
Human Complement C1q	Polyclonal	Goat	FITC	Calbiochem	1:300	30 mins
Human IgG (γ chain specific)	Monoclonal	Goat	PE	Sigma	1:300	40 mins
Human IgM (μ chain specific)	Monoclonal	Goat	FITC	Sigma	1:300	40 mins
Murine IgM (μ chain specific)	Monoclonal	Goat	PE	Sigma	1:300	30 mins
Human Factor H	Polyclonal	Goat	-	Calbiochem	1:300	40 mins
Human Serum Amylase Protein	Polyclonal	Rabbit	-	Calbiochem	1:300	40 mins
Human C-Reactive Protein	Polyclonal	Rabbit	-	Calbiochem	1:300	40 mins
Goat/Sheep IgG	Monoclonal	Donkey	FITC	Serotec	1:300	30 mins
Rabbit IgG	Monoclonal	Goat	FITC	Sigma	1:300	40 mins
Goat IgG	Polyclonal	Rabbit	Immunogold	BB Int	1:200	3 hours

Table 2.5 Antibodies, stains and toxins used in macrophage cell work in this thesis

Target	Monoclonal/ Polyclonal	Host Species	Conjugation	Company	Dilution	Incubation Duration
Murine F4/80	Monoclonal	Rat	PE	Bioreactives	1:100	1 hour
Human/Mouse NFκB RelA (p65)	Polyclonal	Rabbit	-	Santa Cruz	1:100	Overnight (4°C)
IκB-α	Monoclonal	Rabbit	-	NEB	1:3000	Overnight (4°C)
phosphorylated p38 MAPK	Monoclonal	Rabbit	-	NEB	1:3000	Overnight (4°C)
phosphorylated ERK1/2	Monoclonal	Rabbit	-	NEB	1:3000	Overnight (4°C)
β-actin	Monoclonal	Mouse	-	Abcam	1:5000	Overnight (4°C)
Rabbit IgG	Monoclonal	Goat	AF-633	Invitrogen	1:500	1 hour
Rabbit IgG	Polyclonal	Pig	HRP	Santa Cruz	1:3000	1 hour
Mouse IgG	Polyclonal	Rabbit	HRP	Abcam	1:3000	1 hour
DAPI	-		-	Invitrogen	1μg/ml	5 mins

2.5 CELLS

2.5.1 RAW 264.7 Cell Culture

RAW 264.7 (ATCC) cells are derived from a murine macrophage leukaemia cell line. Propagation of cells was conducted in T75 or T175 Nunc flasks at 37^{o}C, 5% carbon dioxide using RPMI 1640 (Invitrogen) with 10% FBS supplement as medium until cells formed a confluent layer. Cells were washed with HBSS without calcium and magnesium, and then trypsinised using with 0.025% trypsin with EDTA solution (BioWhittaker). Cells were viewed under microscope and agitated until the majority of cells were dislodged. An equal volume of media was added to neutralise the trypsin, and the suspension was then washed using HBSS without calcium and magnesium. The pellet was resuspended in 5mls and 2 x 10^5 cells/ml, passaged into a T75 Nunc flask and incubated as above.

2.5.2 Neutrophil Extraction from Human Volunteers

Neutrophils were extracted from blood donations from human volunteers (Segal et al. 1980). 100mls of freshly collected blood was heparinised (300U/50mls) and floated onto Lymphoprep (Axis Shield), followed by centrifugation at 2,000 rpm for 30 mins without brakes to ensure separation into three distinct layers: the upper PBMC layer, a middle layer containing remaining Lymphoprep and neutrophils and the lower sedimented erythrocytes. The monocyte interface was removed and the middle and lower layer pooled to create a final volume of 40mls. 10mls of 10% Dextran (MW 200,000-300,000) (MP Biomedical) in normal saline was added and sedimentation of erythrocytes was allowed to occur for an hour by leaving undisturbed at room temperature. The upper neutrophil containing band was extracted into a fresh container, centrifuged at 2,000 rpm for 10 minutes and remaining

erythrocytes were removed by hypotonic lysis. Neutrophils were counted and assessed for viability using Trypan Blue exclusion and used within 4 hours. In addition 100µl of the cell preparation was diluted 1:100 with PBS, cytospun, stained using Diff-quick (Merck) and 400 cells morphologically assessed under microscopy. Neutrophil extractions were found to be over 95% pure using this method.

2.6 CAPSULE POLYSACCHARIDE METHODS

2.6.1 All-Stains Assay for Acidic Polysaccharides

S. pneumoniae strains were grown overnight on 5% blood Columbia agar plates, inoculated into 15mls Cden medium and grown to OD 0.4. Following 10 mins centrifugation at 4,800 rpm bacteria were resuspended in 500µl ddH_20 and 1ml chloroform was added. After shaking, 400µl of the aqueous layer was added to 2mls All Stains Solution [10mg All Stains (Sigma) in 50mls 50% formamid and 30µl acetic acid]. The optical density of samples was measured at a wavelength of 640nm, with various strains being processed in triplicate separately for analysis.

2.6.2 The Quellung Reaction

S. pneumoniae strains were grown overnight on blood agar plates 37 °C in 5% CO_2 on 5% blood Columbia agar. One loop of each bacterial strain was inoculated into 15mls THY Broth and grown at 37°C in 5% CO_2 until mid-log phase (OD 0.4). One loop of bacterial culture solution was spread on a glass slide; one loop of capsular type specific rabbit anti-serum (Staten's Serum Institute) was mixed and allowed to react for 1 minute. A further

loop of filter sterilised India ink (Winsor and Newton) was added to the mixture, which was placed under a cover-slip and viewed under oil immersion x100 magnification.

2.7 COMPLEMENT FACTOR AND MEDIATORS METHODS

2.7.1 Complement Factor Binding Assays

5 x 10^6 *S. pneumoniae* bacteria were added to each eppendorf, centrifuged at 13,000 rpm for 5 minutes and supernatant aspirated without disturbing the pellet. 10µl PBS, or 25% human serum, was added to each pellet and incubated at 37°C for 20 minutes. Samples were then washed twice using 0.1% PBS Tween-20 and 50µl of appropriate primary antibody added (at a dilution of 1:300 with 0.1% PBS Tween). An incubation of 30 minutes on ice was followed by a further two washes with 0.1% PBS Tween-20. Samples were then fixed with 100µl 3% PFA and 200µl PBS if a directly conjugated antibody was used initially. Samples requiring a secondary antibody were instead subjected to a 30 minute incubation on ice with 50µl conjugated secondary antibody (also at a 1:300 dilution in 0.1% PBS Tween-20), followed by the same washing and fixation process. Samples were then analysed by flow cytometry using FacsCalibur, selecting 25,000 cells for analysis.

2.7.2 Antibody (IgG and IgM) Binding Assays

Total IgG and IgM binding to *S. pneumoniae* was assessed by adapting the protocol for complement binding assays detailed above. An antibody mixture comprised of 1:300 goat anti-human IgM FITC conjugated and 1:300 goat anti-human IgG phycoerythrin conjugated in PBS Tween 0.1% was incubated on ice for 30 minutes, with positive controls stained singly with each antibody. Analysis by flow cytometry included using the singly

probed IgM and IgG bacteria to ensure that there was no bleeding through of either signal on the double probed samples.

2.7.3 Immunoblot for Complement Component 3

S. pneumoniae strains were serially diluted from 1 x 10^8 to 1 x 10^6 CFU, and to each bacterial dilution 100µl 10% human serum was added. Bacteria were incubated for 20 minutes at 37°C, centrifuged for 15 mins at 13,000 rpm and the supernatant carefully transferred to a clean eppendorf. The supernatant was then diluted 1:10 with PBS (giving a final serum dilution of 1%) and 50µl of this solution was added to 10µl laemelli buffer [3.125mM TRIS HCl, pH 6.8, 10% SDS, 20% Glycerol, 50nM DTT, bromophenol blue, 4.375ml H_2O] and incubated for 5 mins at 100°C. 15µl of each sample was loaded onto a 12% acrylamide gel in running buffer [0.25M TRIS Base, 1.92M Glycine, 1% SDS] and run at 100V to separate proteins. Protein bands were then transferred onto a nitro cellulose membrane for 1 hour at 20V using transfer buffer [50mM Tris, 150mM NaCl, 0.1% Tween-20, pH 7.4] and the membrane blocked overnight with TBS-Tween and 5% milk. Following 3 washes with TBS-Tween, membranes were probed for 1 hour with agitation using 1:4000 anti-human C3 antibody conjugated with HRP in a final volume of 5mls TBS-Tween 5% milk. After a further 3 washes with TBS-Tween the membrane was developed with ECL Reagent (Amersham) and exposed onto photographic film (Kodak) for between 1 and 10 minutes.

2.8 ELECTRON MICROSCOPY METHODS

2.8.1 Section Preparation

S. pneumoniae strains were grown overnight on 10% blood Columbia agar plates, inoculated into 30mls THY medium and grown to OD 0.4. Cultures were equally divided in two, and centrifuged at 4,800 rpm for 15 minutes. Pellets were resuspended in 2mls of PBS or 20% human serum and incubated for 30mins at 37°C. Samples were washed twice in PBS and fixed with 1ml 1% PFA for 1 hour at 4°C. The samples were then pelleted again and resuspended in 1.5% low gelling temp agarose (Sigma) in 0.1M phosphate buffer and left overnight at room temperature. The agarose plugs were cut into $1mm^2$ blocks and incubated in a solution of 2% formaldehyde (Sigma), 2.5% glutaldehyde (Sigma), 0.075% ruthenium red (Agar Sci), 0.075M lysine acetate (Sigma) in cacodylate buffer [0.1M cacodylate trihydrate, pH 7.4, 3% sucrose, 0.1% CaCl] on ice for 20 minutes. Samples were washed twice with 0.075% Ruthenium Red in cacodylate buffer and incubated in 2% formaldehyde, 2.5% glutaldehyde, 0.075% ruthenium red in cacodylate buffer for 3 hours on ice. Following a further two washes samples were fixed with 1% osmium in cacodylate buffer containing 0.075% ruthenium red for 1 hour on ice. Samples were washed several times with cacodylate buffer 0.075% ruthenium red and then dehydrated using ethanol (Sigma) (10%, 20%, 50%, 70%, 90%, 100%) for 30 minutes at each concentration in the series on ice. Dehydrated blocks were then incubated in a solution of 1 part LR White Resin (Agar Sci) to 1 part ethanol for 2 hours on ice, and then overnight in 2 parts LR White to 1 part ethanol. Samples were incubated in 100% LR White Resin for 3 days at 4°C with the resin changed every 12 hours. Finally blocks were baked in gelatin capsules in

LR White Resin at 65°C for 24 hours, cut into very fine sections and mounted on copper or nickel grids.

2.8.2 Capsule Thickness Measurement

Sections were stained with 1% uranyl acetate for 3 minutes, washed 10 times in distilled water and then stained with lead citrate [0.19M, Reynold's Lead Citrate Stain] for 3 minutes and again washed 10 times in distilled water. Following air drying sections were viewed using a Jeol 1010 transmission electron microscope (100kV). Images were obtained for at least 10 randomly chosen bacteria for each strain investigated and analysed using Image J software (http://rsb.info.nih.gov/ij). The cross-sectional area of the whole bacterium including and excluding the capsule were obtained and, by assuming circularity, used to calculate the bacterial radius with or without the capsule and hence the average width of the capsule layer. Data is expressed as a mean value for each strain investigated.

2.8.3 C3b/iC3b Immunogold Staining

Sections were inverted onto 50µl 10% rabbit serum (Sigma) and incubated at room temperature for 30 minutes. The sections were then washed twice with PBS and inverted onto either 1/100 goat anti human C3 in PBS or PBS alone overnight at 4°C. Sections were washed 5 times in PBS and then inverted onto 50µl immunogold conjugated rabbit anti-goat IgG for 3 hours at room temperature. Finally grids were washed 3 times in PBS, 5 times in distilled water and air dryed. Sections were stained with lead nitrate as in section 2.8.2 and visualised using a Jeol 1010 transmission electron microscope (100kV). Data was obtained for at least 10 randomly chosen bacteria for each strain investigated and expressed as medians with IQRs.

2.9 IN VITRO CELLULAR METHODS

2.9.1 Neutrophil Opsonophagocytosis Assay

Human serum was diluted in Hanks Buffered Salt Solution (HBSS) with divalent cations and 10µl added per well in a 96 well plate. HBSS with divalent cations and heat-treated serum were used as controls. Bacteria were diluted in HBSS with divalent cations (GIBCO) to optimised multiplicity of infections (MOI), with 10µl of FAM-SE labelled *S. pneumoniae* added per well and incubated for 30 mins at 37°C and 150 rpm. PMNs extracted from human volunteers were washed once in HBSS without divalent cations and once in HBSS with divalent cations whereas neutrophils extracted from human blood were washed in HBSS with divalent cations. Neutrophils were counted using Trypan Blue exclusion, and 5 x 10^5 cells added per well. The opsonised *S. pneumoniae* were incubated with neutrophils for 30 mins, 37°C, 150rpm and the cells fixed with 50µl 3% PFA. Cells were analysed for fluorescence using flow cytometry (FacsCalibur), selecting 15,000 cells for analysis. For experiments using cytochalasin D, neutrophils were incubated with 1µM cytochalasin D (Sigma) for 30 mins at room temperature before incubation with FAM-SE bacteria. Trypan blue quenching was performed by adding 1mg/ml Trypan Blue (Sigma) before samples were analysed by flow cytometry.

2.9.2 RAW 264.7 Cell Opsonophagocytosis Assay

RAW 264.7 cells were trypsinised, counted and adjusted to a concentration of 5 x 10^5 cells/ml. 400µl of the cell suspension was added to each well of 24 well plates and allowed to adhere for 1 hour. FAM-SE labelled *S. pneumoniae* were incubated for 30’ at 37°C in

PBS, 20% HK Serum or 20 % normal serum, washed and resuspended in RAW 264.7 culture medium. Culture wells were aspirated and 400µl of 5 x 10^6 opsonised bacteria was added to each well, giving an MOI of 10 bacteria per RAW 264.7 cell. RAW 264.7 cells were incubated at 37°C with 5% CO_2 for 15 minutes, 1 hour, and 2 hours or 4 hours, after which time the supernatant was removed. Wells were washed three times with HBSS without divalent cations (GIBCO). Cells were trypsinised using 300µl 0.025% trypsin with EDTA solution (BioWhittaker) and 50µl FBS added to each well to neutralise the trypsin. Finally 150µl 3% PFA was added to each well and samples were analysed by flow cytometry using FacsCalibur (Becton Dickinson).

2.9.3 TNFα Time Course

2 x 10^5 RAW 264.7 cells were seeded into each well of 24 well plates (Nunc) and allowed to adhere for 1 hour. FAM-SE labelled *S. pneumoniae* were incubated for 30 minutes at 37°C in PBS, 20% HK Serum or 20 % normal serum, washed and resuspended in RAW 264.7 culture medium. Wells were aspirated; 400µl of culture medium containing opsonised bacteria was added to each well, giving an MOI of 10 bacteria per RAW 264.7 cell. 1ng/ml LPS (lipopolysaccharide) (InvivoGen) was used as a positive control. Cells were incubated at 37°C with 5% CO_2 for 3, 6, and 12 or 24 hours, after which the supernatant was aspirated, centrifuged at 13,000 rpm for 15 minutes to remove bacteria in suspension and stored at -20°C until processing. Samples were analysed using the TNFα ELISA outlined in 2.11.1.

2.9.4 NFkB Translocation Assay

RAW 264.7 cells were trypsinised, counted and adjusted to a concentration of 5 x 10^5 cells/ml. 400µl of the cell suspension was added to each well of 24 well plate (Nunc) containing a pre-sterilised, 13mm diameter glass cover-slip (Invitrogen) and allowed to adhere for 3 hours. Dilutions of *S. pneumoniae* were made in RAW 264.7 culture medium with 25% human serum added to ensure MOI of 1, 10 and 100. These were incubated for 30 minutes at 37°C, centrifuged at 13,000 rpm for 10 mins and resuspended in the original volume of culture medium. 400µl opsonised *S. pneumoniae* or 1ng/ml Ultrapure LPS from *Escherichia coli* O111:B4 (InvivoGen) were added to aspirated RAW 264.7 cell wells and incubated at 37°C for 1 hour. Following the incubation, the supernatant was aspirated; the cover-slips were covered with 3% PFA and left for 15 mins in the dark, washed three times with HBSS with divalent cations (Invitrogen) and resuspended in 400µl HBSS with divalent cations and stored at 4°C in the dark until staining.

Cover-slips were stained by floating face down on 50µl of solution at room temperature with the following method, unless otherwise stated, and washed 3 times in TBS between staining solutions. Cover-slips were washed three times in TBS followed by 50µl 0.2% Triton-X100 (Sigma) for 10 minutes. This was followed by a 30 minute block using 10% goat serum (Sigma) and then 1:100 Rabbit anti-NFκB antibody in 10% goat serum overnight at 4°C. Cover-slips were then floated face down on 1:500 Alexa Fluor 633 conjugated goat anti-rabbit IgG in 10% goat serum for 1 hour. Cover-slips were mounted using Vectashield containing DAPI (Vector Labs) and analysed using confocal microscopy.

2.9.5 Confocal Microscopy Image Acquisition

Images were acquired using a Leica SP2 confocal microscope, with 5 frames taken for each stain analysis. Each fluorochrome was captured using a sequential acquisition with a pin hole of 1 Airy, scan speed of 400Hz and 4 frame averaging used. Fluorochromes used were DAPI (excitation 405 nm, emission 400–450 nm), AF555 (excitation 543 nm, emission 560–580 nm), AF633 (excitation 633 nm, emission 650–700 nm), FITC (excitation 488nm, emission 515-545nm) and PE (excitation 488nm, emission 560-580nm). A sub-saturating fluorescence intensity with optimal signal to noise ratio was achieved by adjusting the photomultiplier tube offset and gain. Each field was selected based on identification of a near confluent cell layer by identifying nuclear (DAPI) staining (Noursadeghi et al. 2008) .

Image analysis was performed with Metamorph v7.17 (Molecular Devices) to quantify nuclear:cytoplasmic ratios NF-κB RelA staining and proportion of cells demonstrating positive co-localization of DAPI/RelA (AF655) staining (correlation coefficient >0.5) as markers of NF-κB nuclear translocation (Noursadeghi et al. 2009).

2.9.6 Innate Activation Westerns

1 x 10^6 RAW 264.7 cells were added to each well of 6 well plate (Nunc) and allowed to adhere for 1hr at 37°C. Dilutions of *S. pneumoniae* were made in RAW 264.7 culture medium with 25% human serum added to ensure MOI of 10, incubated for 30 minutes at 37°C, centrifuged at 13,000 rpm for 10 mins and resuspended in the original volume of culture medium. 400µl opsonised *S. pneumoniae* or 1ng/ml LPS were added to aspirated RAW 264.7 cell wells and incubated at 37°C for 15, 30, 60 or 120 minutes. Supernatant was aspirated and cell lysates were collected in into SDS sample buffer [62.5 mM Tris-HCl

(pH 6.8), 10% glycerol, 2% SDS, 0.01% bromphenol blue, and 5% 2-ME], sonicated, and heated (100°C for 5 min) before PAGE (4–12% gradient gels) and transfer onto Amersham Hi-bond membranes (GE Healthcare). Membranes were blocked for 1 hour in 5% milk powder in TBS with 0.05% Tween 20 (Sigma-Aldrich) and then immunoblotted sequentially with primary antibody overnight (4°C), and HRP-conjugated secondary antibody for 1 hour (room temperature), all prepared in TBS/Tween 20 with 1% milk powder. Membranes were washed with TBS/Tween-20 after each step. Immunostains were developed with Amersham ECL reagent (GE Healthcare) and visualized on Amersham Hyperfilm ECL (GE Healthcare), according to manufacturer's instructions. Antibodies used in these westerns are listed in Table 2.5.

2.10 IN VIVO METHODS

2.10.1 Pneumonia Infection Model

5 x 10^5 colony forming units of FAM-SE labelled *S. pneumoniae* in filtered sterilised PBS was inoculated intranasally (IN) into mice anaesthetised using a halothane chamber. During inoculation the mouth was held closed to ensure that all inspiration was through the nose to promote optimum uptake of inoculums into the lungs. After 4 hours mice were culled with an overdose of pentobarbitone and the diaphragm carefully cut to allow lung expansion. The neck was dissected to reveal the trachea and a cannula inserted through a small opening made with a scalpel. 1ml filter sterilised PBS was flushed three times before collection of the bronchoalveolar lavage fluid (BALF).

50µl aliquots of BALF were immediately frozen at -80C and cytokine levels analysed later (see 2.9). BALF was also plated undiluted and at 1/10 and 1/100 dilutions on 5% blood Columbia agar plates with 5mg/ml gentamycin (Sigma), which were incubated overnight at 37°C and *S. pneumoniae* colonies counted to determine free bacterial counts. Furthermore, alveolar macrophages were stained with anti-mouse F4/80 phycoerythrin conjugate antibody and this population isolated using flow cytometry (FacsCalibur), and the association of FAM-SE stained bacteria was determined in this cell population.

2.10.2 Intraperitoneal (IP) Model

Mice were inoculated by intraperitoneal injection with 2000 CFU *S. pneumoniae* in a 100µl volume. Organs were harvested 24 hours after inoculation and blood collected by terminal cardiac puncture under pentobarbitone anaesthesia. Serial dilutions of either blood or splenic homogenates were plated onto blood agar to allow calculation of bacterial CFU within the spleen and blood of each mouse.

2.10.3 Competitive Index (CI) Method

Mixtures of the two *S. pneumoniae* strains being compared were inoculated in a 1:1 ratio, with the inoculum diluted and plated onto plain and antibiotic medium to determine the ratio of the strains using antibiotic sensitivities to discriminate between strains. Mice were inoculated by intraperitoneal injection with a total of 5×10^4 CFU, organs harvested (blood and spleen) extracted as previously described after 24 hours. Dilutions of blood and splenic homogenates were plated onto plain and antibiotic medium to determine the ratios of the two strains using antibiotic sensitivities. The relative virulence of the strains was calculated as a competitive index (CI), defined as the ratio of the test strain (unencapsulated strain) to

the reference strain (WT encapsulated strain) recovered from the mice divided by the ratio of the test strain to the reference strain in the inoculum (Brown et al. 2001). A CI of <1.0 indicates that the test strain is reduced in virulence compared with the reference strain, with the lower the CI the greater the reduction in virulence.

2.11 CYTOKINE METHODS

2.11.1 TNFα ELISA

ELISAs were performed using a murine TNFα kit (RnD Systems) Briefly ELISA plates were coated overnight at room temperature with 50µl 144µg/ml capture antibody (goat anti-mouse TNFα) in PBS. Following aspiration and three washes with wash buffer [0.05% Tween-20 in PBS], 300µl 1% BSA in filter sterilised PBS was added to each well and allowed to incubate for 1 hour at room temperature. Three further washes were performed using wash buffer and samples were diluted to appropriate concentrations predetermined in pilot experiments in reagent diluent [1% BSA in filter sterilised PBS]. 50µl sample or standard was added per well and incubated for 2 hours at room temperature. Three more washes with wash buffer were performed and 50µl 27µg/ml detection antibody (biotinylated goat anti-mouse TNFα) (in reagent diluents) was added to each well, incubated for 2 hours and 3 further washes performed. Finally 50µl 1:200 Streptavidin-HRP conjugated antibody was added to each well, incubated for 20 minutes in the dark at room temperature and the reaction stopped by adding 25µl 0.19M H_2SO_4. Samples were analysed using a plate reader at 550nm wavelength.

2.12 STATISTICS

Statistical analysis was performed using Prism Version 4.0. Results expressed as means were compared between strains using one way ANOVAs with post-hoc tests. Results presented as medians (IQRs) were compared using the Kruskal Wallis test with Dunn's multiple comparison test (multiple groups) or the Mann Whitney U test (for two groups). Data are representative of results obtained with repeated assays with at least three replicas per experimental condition.

CHAPTER 3

ROLE OF CAPSULE IN COMPLEMENT DEPOSITION AND NEUTROPHIL INTERACTIONS

3.1 INTRODUCTION

Although unencapsulated *S. pneumoniae* strains are never isolated from invasive disease, clearly outlining the importance of the capsule in pneumococcal virulence, there is a surprising lack of detail known about exactly how the capsule assists the development of infection. Previous studies have shown that that deposition of the complement opsonins C3b/iC3b is increased on an otherwise isogenic unencapsulated serotype 2 strain (Quin et al. 2007) and capsular serotype affects the site and nature of complement deposition on *S. pneumoniae* (Winkelstein 1981; Abeyta et al. 2003). The key importance of complement for host immunity to *S. pneumoniae* (Brown et al. 2002; Jonsson et al. 2005; Yuste et al. 2005; Yuste et al. 2008) suggests the effects of the capsule on complement-mediated immunity may be crucial for its role in virulence. However, the mechanisms by which complement deposition on *S. pneumoniae* is prevented by the capsule, which complement pathways are involved and the variation in the complement deposition pattern on encapsulated and unencapsulated *S. pneumoniae* strains are poorly understood. Furthermore, previous data has shown that phagocytosis of *S. pneumoniae* is mainly complement dependent (Wright et al. 1903; Brown et al. 2002; Yuste et al. 2005). Hence the capsule may modulate its effect on phagocytosis solely through its ability to prevent complement deposition or alternatively the capsule could inhibit phagocytosis receptors as well. Overall, there are few data on which effects of the capsule are relevant during infection, and the relative importance during systemic infection of inhibition of

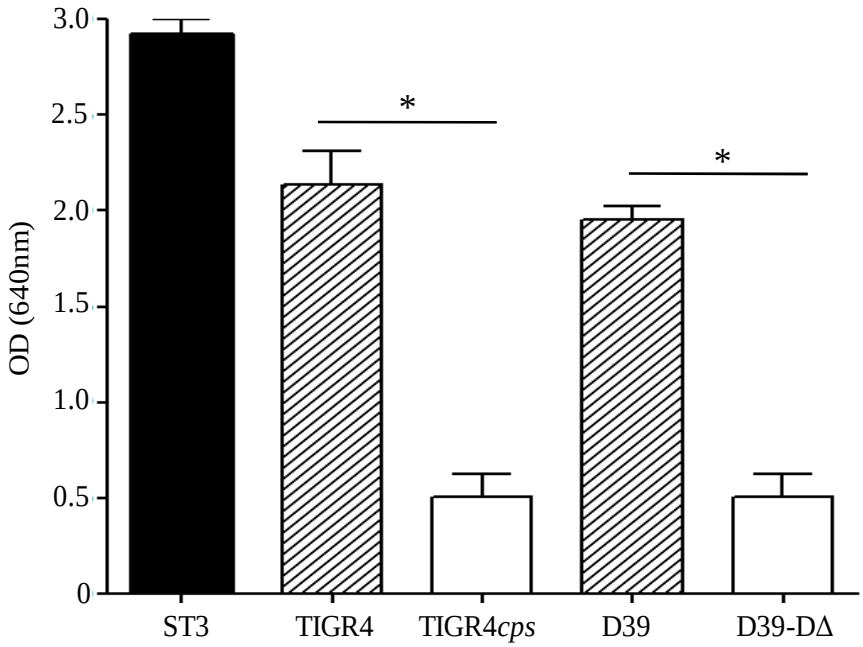
complement-mediated immunity to *S. pneumoniae* compared to other effects of the capsule during systemic infection remains unclear.

In this chapter I have used unencapsulated mutants from serotype 2 (D39-DΔ) and 4 (TIGR*cps*) *S. pneumoniae* strains which are otherwise isogenic to the encapsulated parental (wild-type) strain to investigate the effect of capsule on complement activity and on interactions with neutrophils. In addition, using genetically modified mice I have assessed whether the effects of the capsule on complement pathway activity contributes towards how the capsule increases *S. pneumoniae* virulence during invasive infection.

3.2 RESULTS

3.2.1 Confirmation of capsule depletion in unencapsulated mutants

Unencapsulated mutants from parental strains D39 (serotype 2) and TIGR4 (serotype 4) *S. pneumoniae* strains were confirmed to have the correct mutation and be otherwise isogenic to the encapsulated parental (wild-type) strain in the laboratories which made the mutants (Trzcinski et al. 2003; Morona et al. 2004; Nelson et al. 2007). Quellung reactions were performed on all strains to confirm the serotype of the parental strain and lack of capsule in the unencapsulated mutants (data not shown). In order to confirm the capsule locus mutations in the unencapsulated D39 and TIGR4 *S. pneumoniae* stains caused a change in expression of polysaccharide capsule, a colorimetric assay for the determination of mucopolysaccharides was used (Edstrom 1969). Both the unencapsulated strains of TIGR4 and D39 show very low levels of staining for polysaccharides compared to their encapsulated parental strains (Fig 3.1, $P < 0.001$). We measured the thickness of the capsule after the *S. pneumoniae* strains were fixed and viewed by electron microscopy using a lysine acetate and ruthenium red protocol to preserve polysaccharides ((Hammerschmidt et al. 2005). A serotype 3 strain was used as a positive control, since this serotype is known to have a relatively thick polysaccharide capsule (Hammerschmidt et al. 2005). In confirmation of the results obtained by the colorimetric Stains-All assay (Fig 3.1) both the TIGR4*cps* and D39-DΔ strains were found to have minimal capsule polysaccharide expression, with an average thickness of 12 ± 4nm and 24 ± 9nm respectively (Fig 3.2). The TIGR4 parental strain was found to have a thicker capsule (185 ± 19nm) than the D39 wild-type strain (104 ± 11nm).



<u>Fig 3.1</u> Biochemical capsule assessment in TIGR4 and D39 strains
All Stains Assay semi-quantitative assessment of the amount of bacterium-associated capsule polysaccharide in serotype 3 WT, and TIGR4 and D39 encapsulated (slashed bars) and unencapsulated (open bars) strains (Unpaired student's t-test * $P < 0.001$).

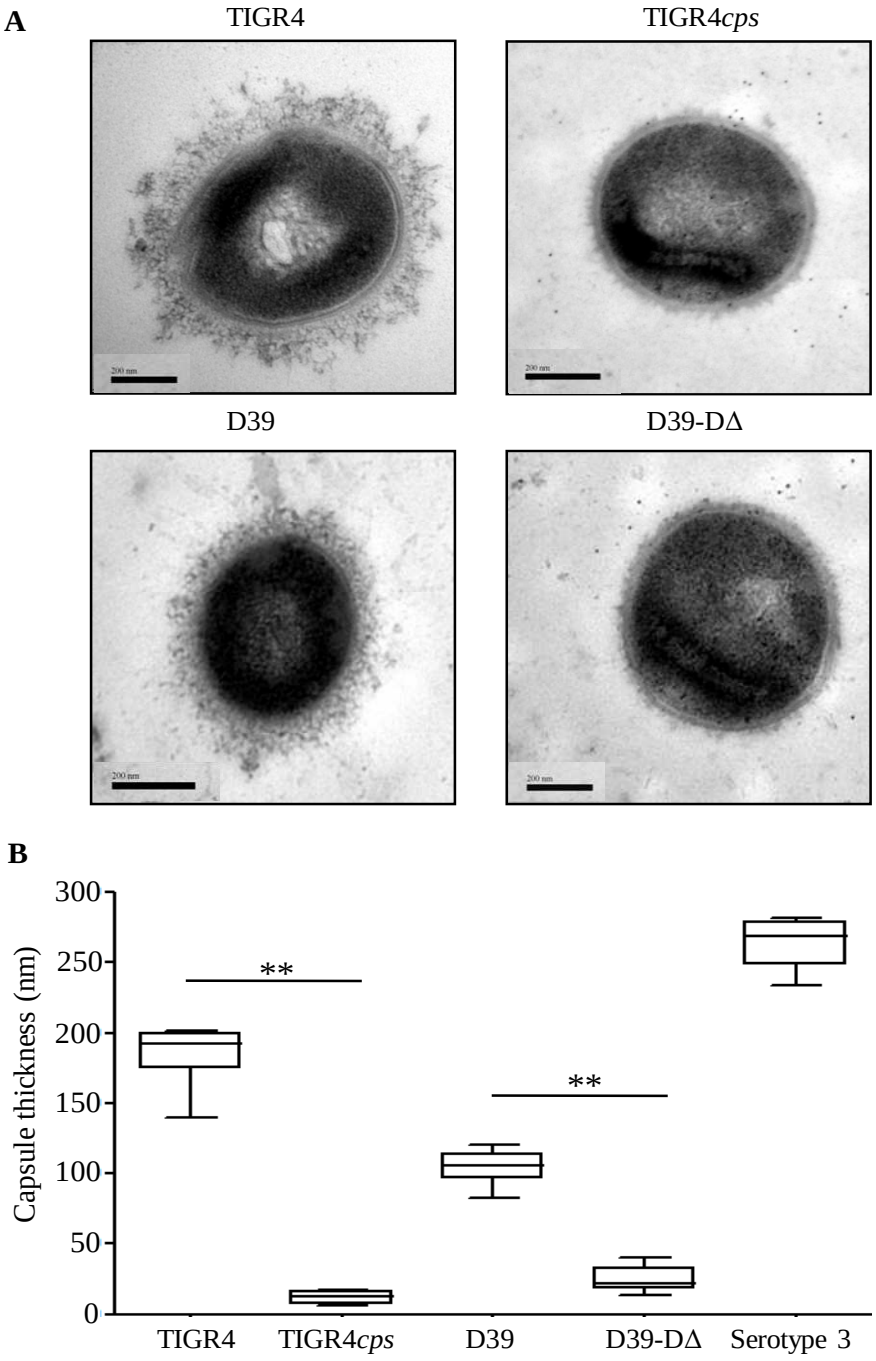


Fig 3.2 EM capsule measurement in TIGR4 and D39 strains

(A). Electron microscopy images (100,000X) of TIGR4, TIGR4*cps*, D39 and D39-DΔ strains when prepared using the LRR fixation method. (B). Capsule thickness as calculated by measuring the area inside the cell wall and the total bacterial area and deriving the radius of the capsule using area = Πr^2. ** $P < 0.001$ (Kruskal-Wallis with Dunn's multiple comparison). The TIGR4 capsule is significantly thicker than the D39 ($P < 0.001$) and both are thinner than that of the serotype 3 strain ($P < 0.001$).

3.2.2 Growth of unencapsulated mutants compared to parental strains

Previous research has indicated that the first four capsule genes are important in growth of the D39 *S. pneumoniae* strain *in vitro* (Battig et al. 2007). In contrast to the results published by Battig et al, there was no detectable growth deficit in our D39-DΔ strain growth in THY broth (Fig 3.3 A and B) compared to the encapsulated wild-type D39. There was also no growth deficit exhibited by the TIGR4*cps* strain in comparison to the wild-type TIGR4. In addition to growth in culture medium, bacterial growth in physiological fluids was determined using human blood or serum. There was no difference in growth in human blood between the unencapsulated or wild-type strains in either the TIGR4 or D39 background, with all four strains growing to approximately 1.90×10^9 cfu/ml after 8 hours (Fig 3.3 C). Bacterial growth in serum also showed no growth deficit between the encapsulated and unencapsulated strains (Fig 3.3 D). Since growth was determined in blood and serum from the same donors, it was possible to compare growth between both fluids as there is no interdonor variability. *S. pneumoniae* growth in serum was significantly greater than in human blood at both the 4 and 6 hour time point (ANOVA $P < 0.001$), perhaps reflecting the effect of immune cells in blood which were not present in human serum. However, all four strains grew to approximately the same level (2.08×10^9 cfu/ml) after 8 hours in both blood and serum. Overall, these results indicate that there is no growth deficit in the unencapsulated mutant in relevant media used throughout this thesis.

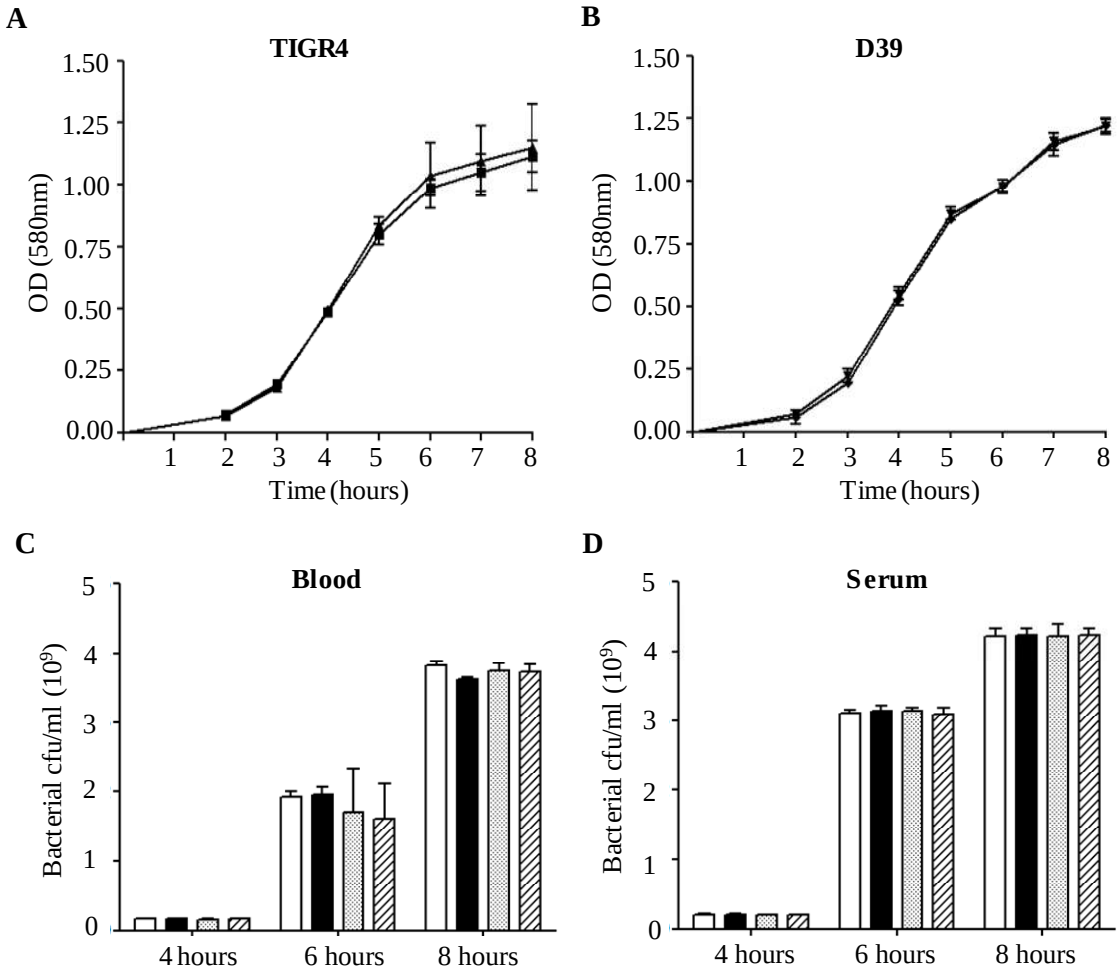


Fig 3.3 Growth of parental and unencapsulated strains

(A), (B). Growth curves of the TIGR4 and D39 strains (square symbols ■) and the TIGR4*cps* and D39-DΔ (triangle symbols ▼) strains respectively in THY respectively. (C), (D). Bacterial growth in human blood and serum for TIGR4 WT (open bars), TIGR4*cps* (black bars), D39 WT (spotted bars) and D39-DΔ (slashed bars). There is no statistically significant difference between the unencapsulated and encapsulated strains.

3.2.3 C3b/iC3b deposition on encapsulated and unencapsulated TIGR4 and D39 in human serum

Using an established flow cytometry assay (Brown et al. 2002; Yuste et al. 2006; Yuste et al. 2007; Yuste et al. 2008), the deposition of C3b/iC3b on *S. pneumoniae* TIGR4 and D39 encapsulated and unencapsulated bacteria was measured in increasing concentrations of human serum pooled from 3 healthy volunteers. Bacteria incubated in PBS alone were used to determine the background fluorescence for each *S. pneumoniae* strain used, and this was repeated in each experiment carried out throughout this thesis. Experiments were repeated using two or more different stock sources for each strain to ensure consistent results. For all of the *S. pneumoniae* strains examined, there was a dose response of C3b/iC3b deposition in relation to human serum (Fig 3.4 and 3.5), with significantly more C3b/iC3b deposited on the bacteria in 100% serum than in all other serum concentrations tested. However, in comparison to the encapsulated wild-type strains, there was a significant increased in C3b/iC3b deposition on both the TIGR4*cps* (Fig 3.4, ANOVA $P<0.001$) and D39-DΔ (Fig 3.5, ANOVA $P<0.001$) *S. pneumoniae* strains. These results indicate that the polysaccharide capsule has a role in preventing complement deposition on *S. pneumoniae* and that this effect is not limited to a single capsule serotype. Interestingly, the extent to which the capsule in prevented complement deposition varied between the D39 and TIGR4 strains, with a larger difference in the results of the C3b/iC3b deposition on the TIGR4 *cps* compared to the TIGR4 strain than between the D39-DΔ and D39 strains. This difference in effect may be due to the effect of capsule serotype, other genetic variation between the strains, or a difference in the expression of capsule polysaccharide i.e. capsule thickness (see Fig 3.1 and 3.2).

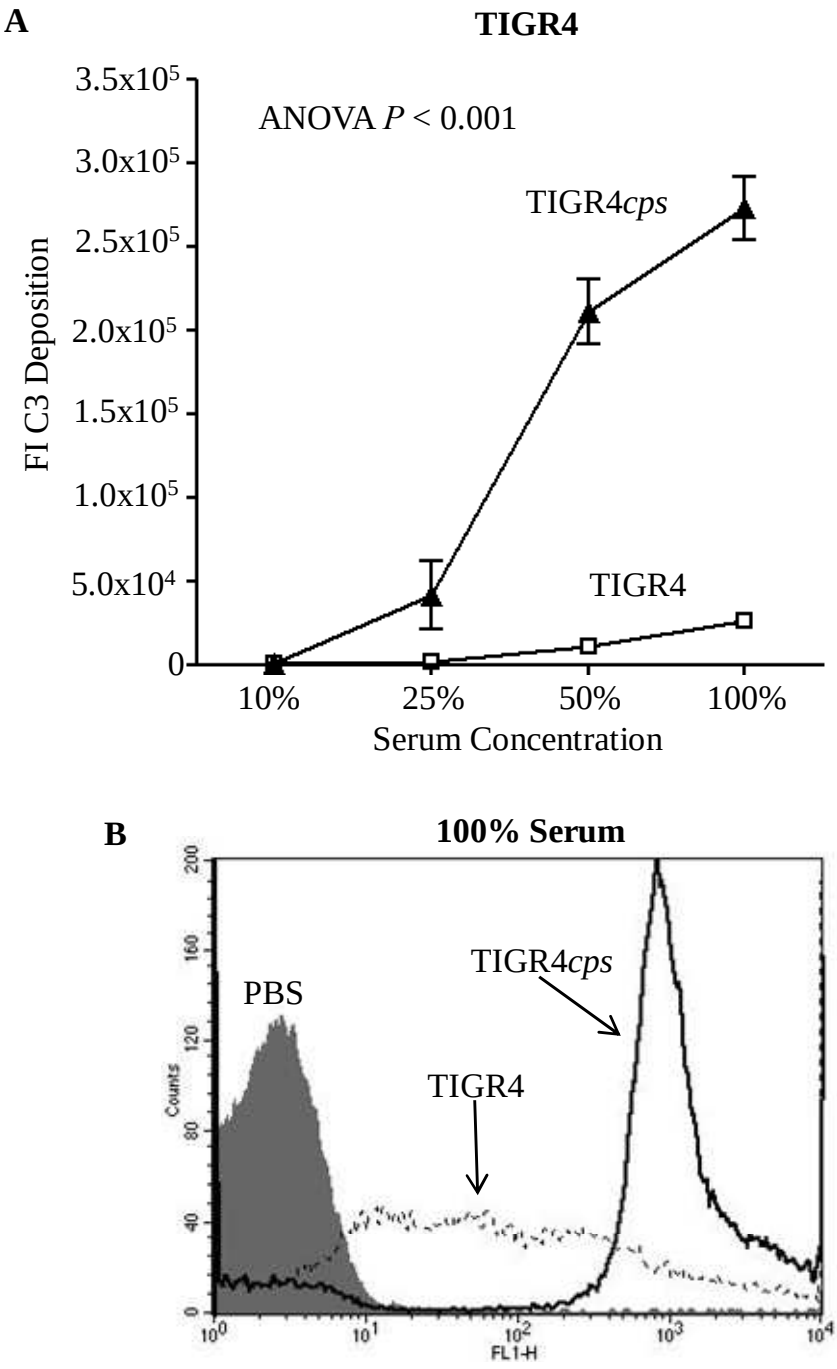


Fig 3.4 C3b/iC3b deposition on TIGR4 strains opsonised with human serum

(A). C3b/iC3b deposition on TIGR4 (triangle symbols ▲) and TIGR4*cps* (square symbols □) strains in different dilutions of human serum, as measured by flow cytometry (ANOVA $P < 0.001$). (B). Representative flow cytometry histogram of C3b/iC3b deposition on TIGR4 encapsulated (solid line) and TIGR4*cps* (dashed line) strains in 100% human serum. Grey represents the results for bacteria incubated in PBS alone.

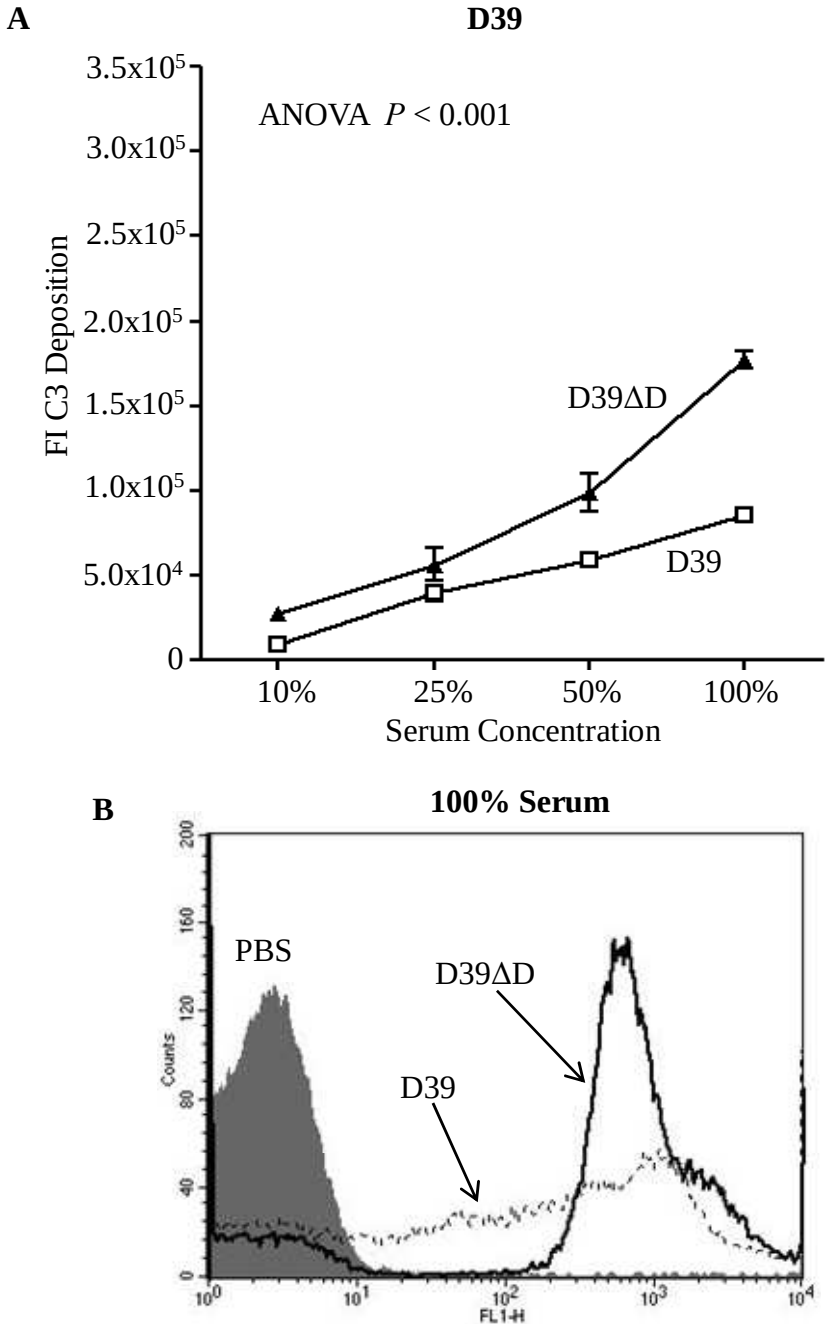


Fig 3.5 C3b/iC3b deposition on D39 strains opsonised with human serum

(A). C3b/iC3b deposition on D39 (triangle symbols ▲) and D39-DΔ (square symbols □) strains in different dilutions of human serum, as measured by flow cytometry (ANOVA $P < 0.001$). (B). Representative flow cytometry histogram of C3b/iC3b deposition on D39 encapsulated (solid line) and D39-DΔ (dashed line) strains in 100% human serum. Grey represents the results for bacteria incubated in PBS alone.

In order to investigate if the increase in C3b/iC3b deposition was due to increased C3b or iC3b deposition on the bacterial cell surface or a combination of the two, the previous flow cytometry assay was repeated using an antibody specific for iC3b alone. For both the TIGR4*cps* and the D39-DΔ strains there was a significantly greater proportion of iC3b deposited on the bacterial cell surface as a proportion of the total C3b/iC3b deposition compared to the relevant parental strain ($P < 0.01$, Fig 3.6, Table 3.1), suggesting that the capsule specifically prevents breakdown of C3b deposited on the *S. pneumoniae* surface to iC3b.

Table 3.1: Relative proportion of iC3b deposited on TIGR4, TIGR4*cps*, D39 and D39-DΔ strains, expressed as a percentage of the total FI C3b/iC3b deposition as measured by flow cytometry.

Strain	Percentage iC3b of total C3b/iC3b	Standard Deviation	*P* value
TIGR4	25.23	0.6	
TIGR4*cps*	44.19	10.4	< 0.01
D39	23.98	1.1	
D39-DΔ	31.70	2.6	< 0.01

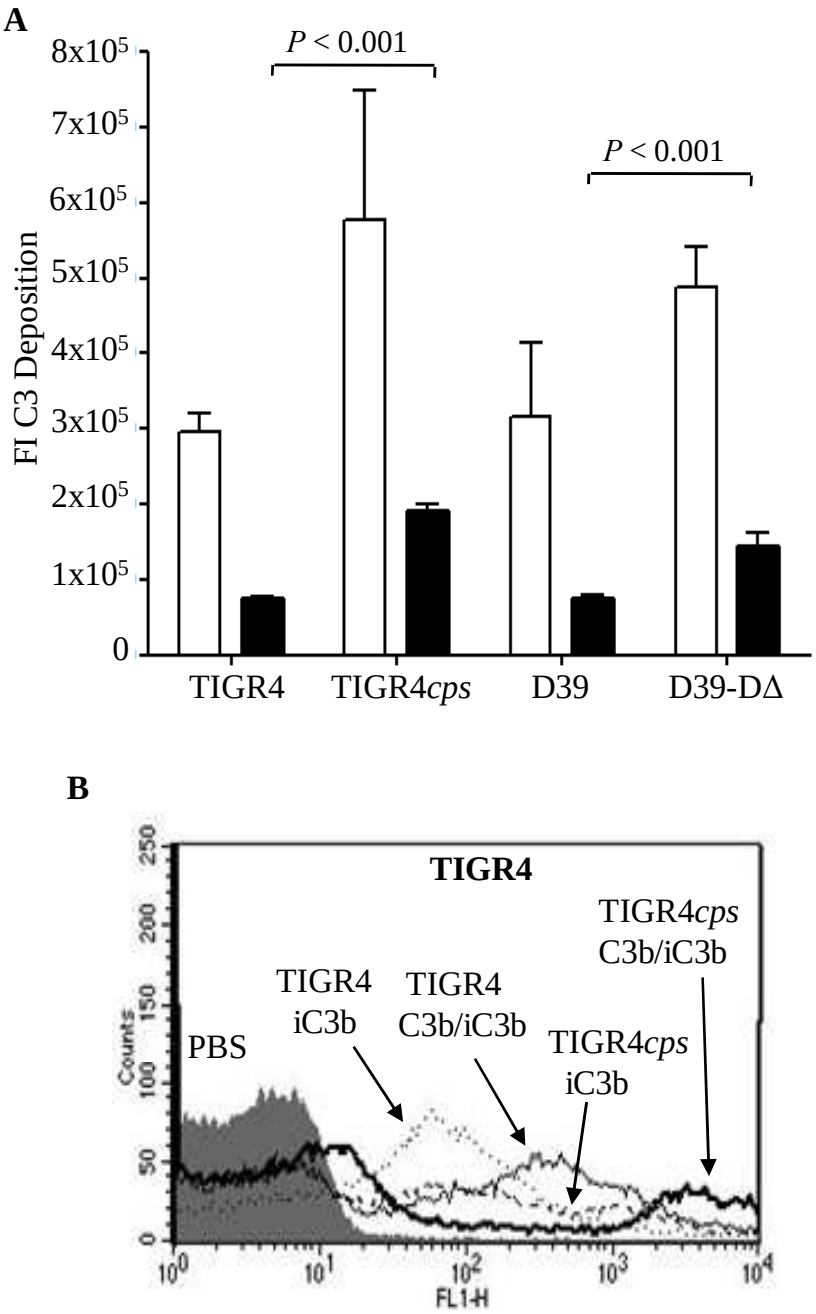


Fig 3.6 C3b/iC3b and iC3b deposition on *S. pneumoniae* strains
(A). C3b/iC3b (white bars) and iC3b alone (black bars) deposition on the TIGR4 and D39 strains and the TIGR4*cps* and D39-DΔ strains in 25% human serum, as measured by flow cytometry (ANOVA $P < 0.001$). (B). Representative flow cytometry histogram of C3b/iC3b and iC3b deposition alone on TIGR4 encapsulated and unencapsulated strains.

3.2.4 Immunoblot against complement component C3 in human serum incubated with *S. pneumoniae* strains

Although the polysaccharide capsule has been shown to not prevent antibody binding to cell wall associated structures such as PspA and PspC (Briles et al. 1981; Lu et al. 2006; Khandavilli et al. 2008), it is possible that the capsule could restrict antibody access to cell surface bound C3b/iC3b. This effect could account for some of the reduction in C3b/iC3b deposition on the encapsulated strains when analyzed using flow cytometry methodology. To confirm that the apparent increase in C3b/iC3b was not due to an artefact created by greater access of anti-C3b/iC3b antibody to the bacterial cell surface in the absence of the capsule, an immunoblot against C3 was used to assess complement activation in serum. A range of doses of encapsulated wild-type or unencapsulated mutant strain were incubated in 10% serum at 37°C for 20 minutes, before probing with an antibody that recognises C3 and all of its breakdown products. Serum incubated in the absence of bacteria was used as a control, and βC3 acts as an internal control in the immunoblot. The immunoblot showed that there were greater quantities of the C3 breakdown products iC3b and C3d in sera incubated with unencapsulated bacteria for both the TIGR4 and D39 strains than in sera incubated with the wild-type strains (Fig 3.7). These results support the flow cytometry assay data and confirm that in the absence of the capsule there is greater activation of complement by *S. pneumoniae*.

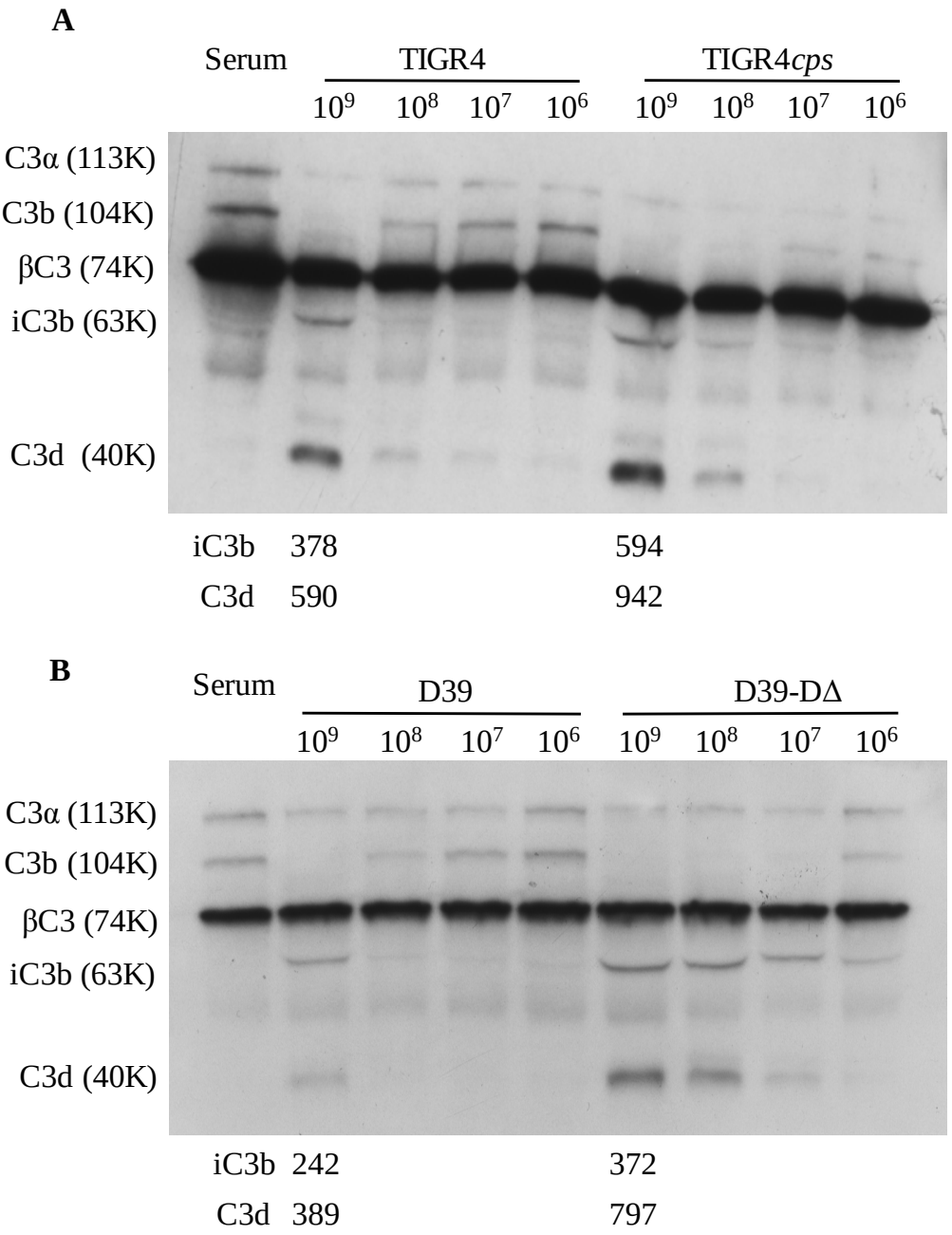


Fig 3.7 Immunoblots for C3 in human serum incubated with *S. pneumoniae*
(A), (B) Immunoblots for complement component 3 when 10% human serum was incubated with TIGR4 (A) and D39 (B) *S. pneumoniae* strains, and then further diluted to a final loading concentration of 1%. βC3 remains constant in serum and acts as an internal loading control. Serum incubated without bacteria was used as a negative control. Densitometry results for iC3b (113K) and C3d (40K) bands for serum incubated with 1 x 10^9 CFU *S. pneumoniae* are listed beneath the relevant lanes.

3.2.5 The capsule inhibits both the classical and alternative complement pathways

In order to determine which complement pathway was responsible for the relatively high level of C3b/iC3b deposition on the unencapsulated strains, the flow cytometry assays were repeated using commercial serum depleted of either C9 (a terminal complement component), C1q (an essential classical pathway mediator) or factor B (Bf) (an essential alternative pathway mediator). For both the TIGR4*cps* (Fig 3.8) and D39-DΔ (Fig 3.9) strain removal of either classical or alternative pathway activity lead to a large decrease in total C3b/iC3b deposition on the bacterial surface, indicating that CPS protects *S. pneumoniae* against the action of both complement pathways.

Further flow cytometry experiments carried out in which the *S. pneumoniae* strains were incubated in serum from C57BL/6 mice confirmed that there was also more C3b/iC3b deposition on the TIGR*cps* and D39-DΔ strains in mouse serum (Fig 3.10 and 3.11). Since the mice have had no prior exposure to pneumococcus, this effect is independent of specific IgG against *S. pneumoniae* antigens. As expected, there was negligible C3b/iC3b deposition on bacteria incubated in $C3^{+/-}$ C57BL/6 mouse serum, confirming the specificity of the assay in detecting C3b/iC3b deposited on bacteria. As expected, there was also markedly reduced C3b/iC3b deposition on the bacterial surface when *S. pneumoniae* were opsonised with mouse sera obtained from mice genetically modified $C1qa^{-/-}$ (lacking C1q and hence classical pathway activity) or $Bf^{-/-}$ (lacking factor B and hence alternative pathway activity) C57BL/6 compared to results for serum from wild-type mice (Fig. 3.9, $P < 0.001$ ANOVA with post-hoc analysis). TIGR4*cps* incubated in serum from either $C1qa^{-/-}$ or $Bf^{-/-}$ C57BL/6 mice showed increased C3b/iC3b deposition compared to the parental TIGR4 strain. D39 *S. pneumoniae* showed a similar pattern of results as the TIGR4 strain

(Fig 3.11), however in keeping with previous results there was a less marked increase in C3b/iC3b deposition on the D39-DΔ strain.

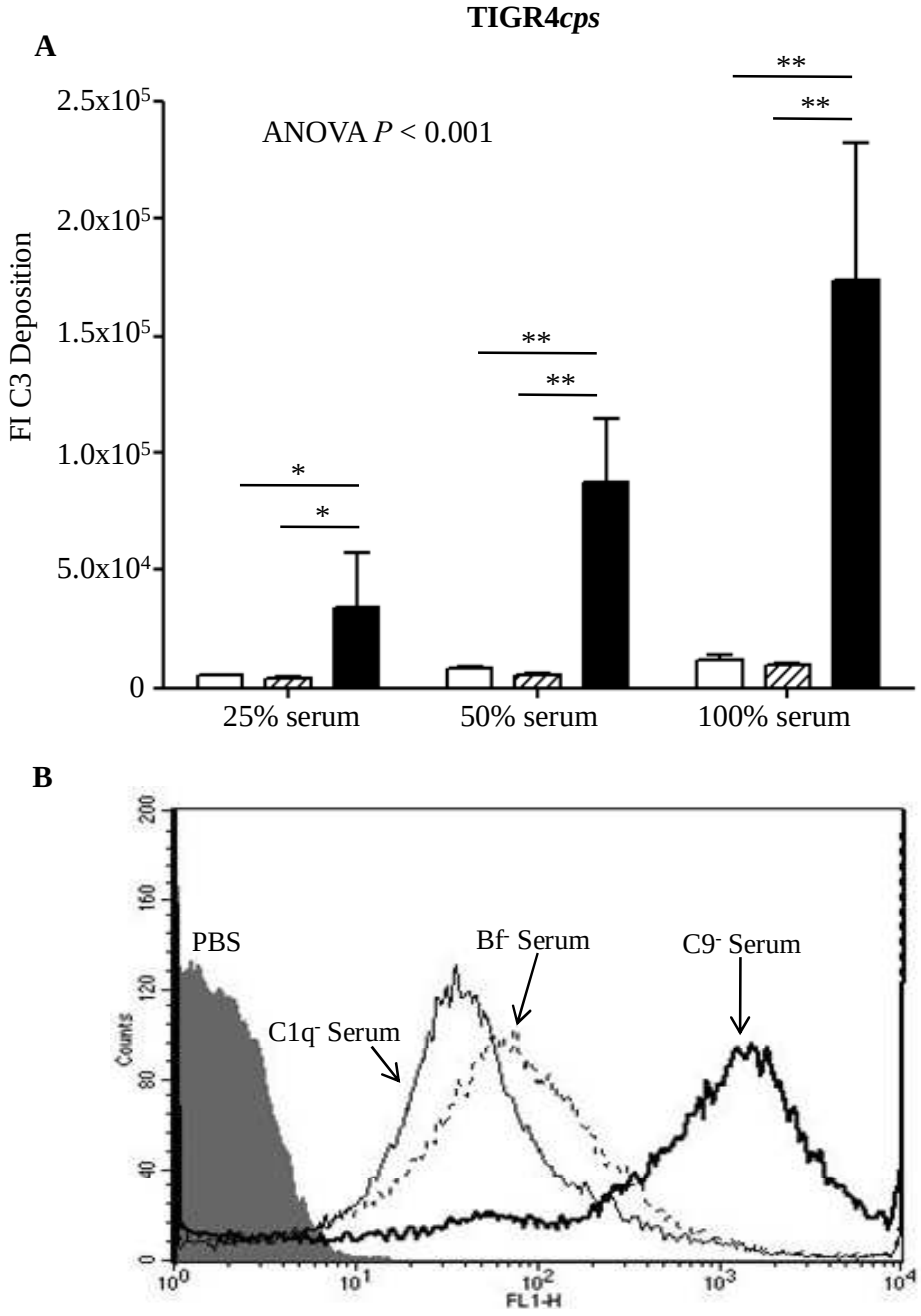


Fig 3.8 C3b/iC3b binding on TIGR4*cps* in complement depleted human serum (A). C3b/iC3b deposition on TIGR4*cps* strain when incubated in human serum depleted of C9 (solid bars), C1q (open bars) or factor B (slashed bars). Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests). (B). Representative flow cytometry histogram of C3b/iC3b deposition on TIGR4 unencapsulated strain in 100% human serum depleted of C9 (thick solid line), C1q (thin solid line) or factor B (dashed line).

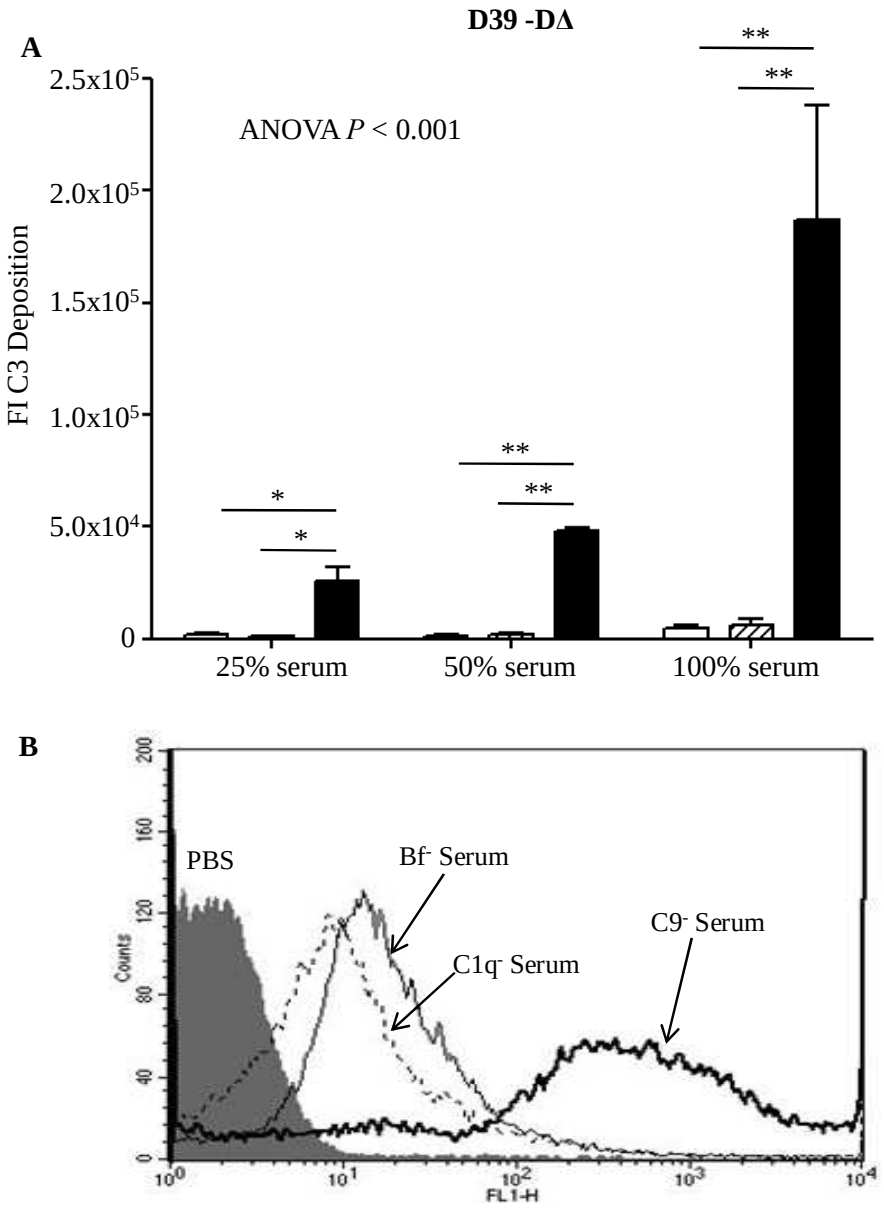
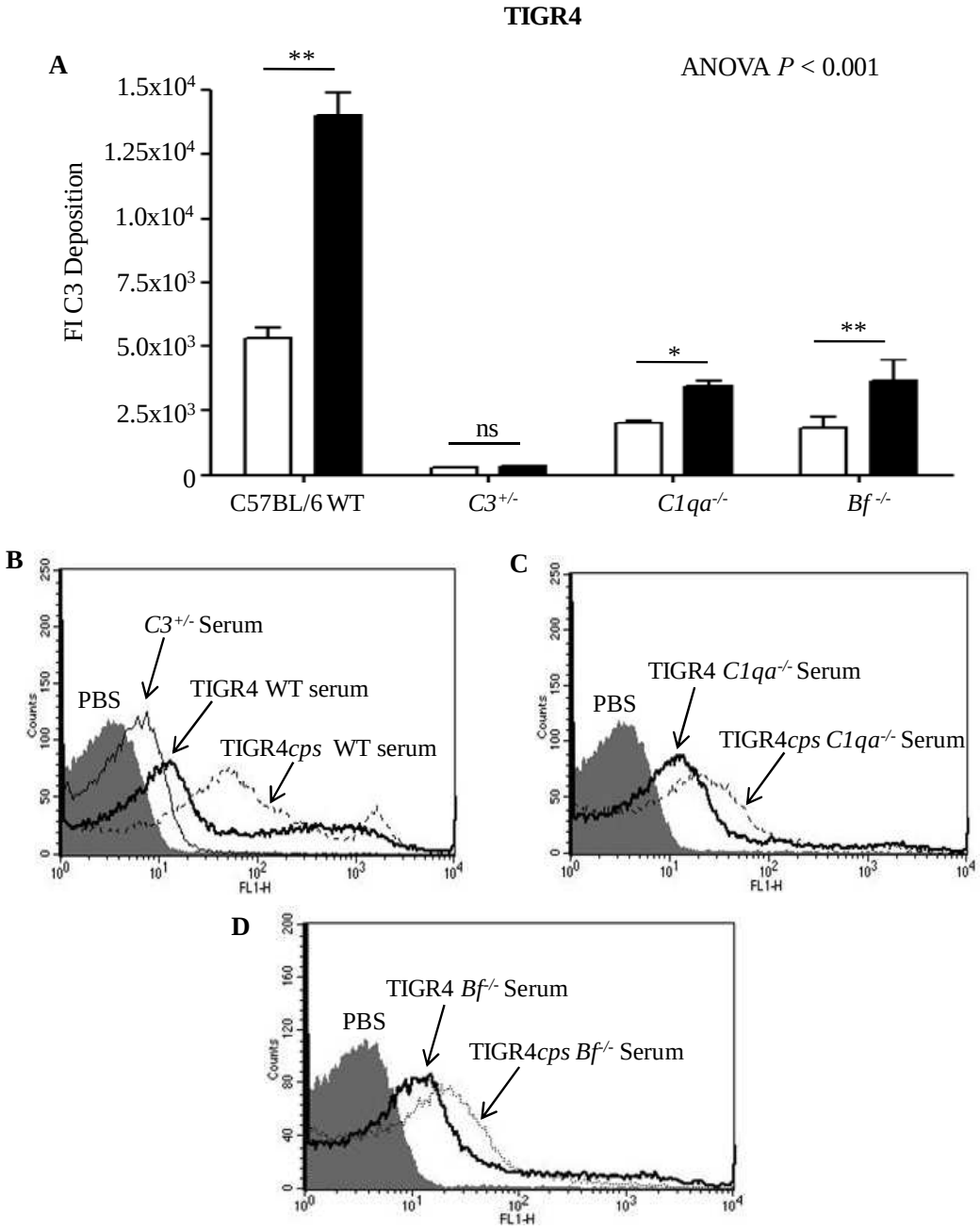


Fig 3.9 C3b/iC3b binding on D39-DΔ in complement depleted human serum
(A). C3b/iC3b deposition on D39-DΔ strain when incubated in human serum depleted of C9 (solid bars), C1q (open bars) or factor B (slashed bars). Error bars represent SDs, * $P <$ 0.01, ** $P <$ 0.001 (ANOVA with post-hoc tests). (B). Representative flow cytometry histogram of C3b/iC3b deposition on D39 unencapsulated strain in 100% human serum depleted of C9 (thick solid line), C1q (thin solid line) or factor B (dashed line).



<u>Fig 3.10</u> C3b/iC3b deposition on TIGR4 strains opsonised in mouse serum

(A) C3b/iC3b deposition on TIGR4 encapsulated (open bars) and unencapsulated (solid bars) strains incubated in 10% serum from C57BL/6 wild-type (WT), *Bf*$^{-/-}$, *C1qa*$^{-/-}$ or *C3*$^{+/-}$ mice. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post hoc tests). (B), (C), (D) Representative flow cytometry histogram of C3b/iC3b deposition on TIGR4 strains incubated in 10% serum from C57BL/6 WT or *C3*$^{-/-}$ (B), *C1qa*$^{-/-}$ (C) and *Bf*$^{-/-}$ (D) mice.

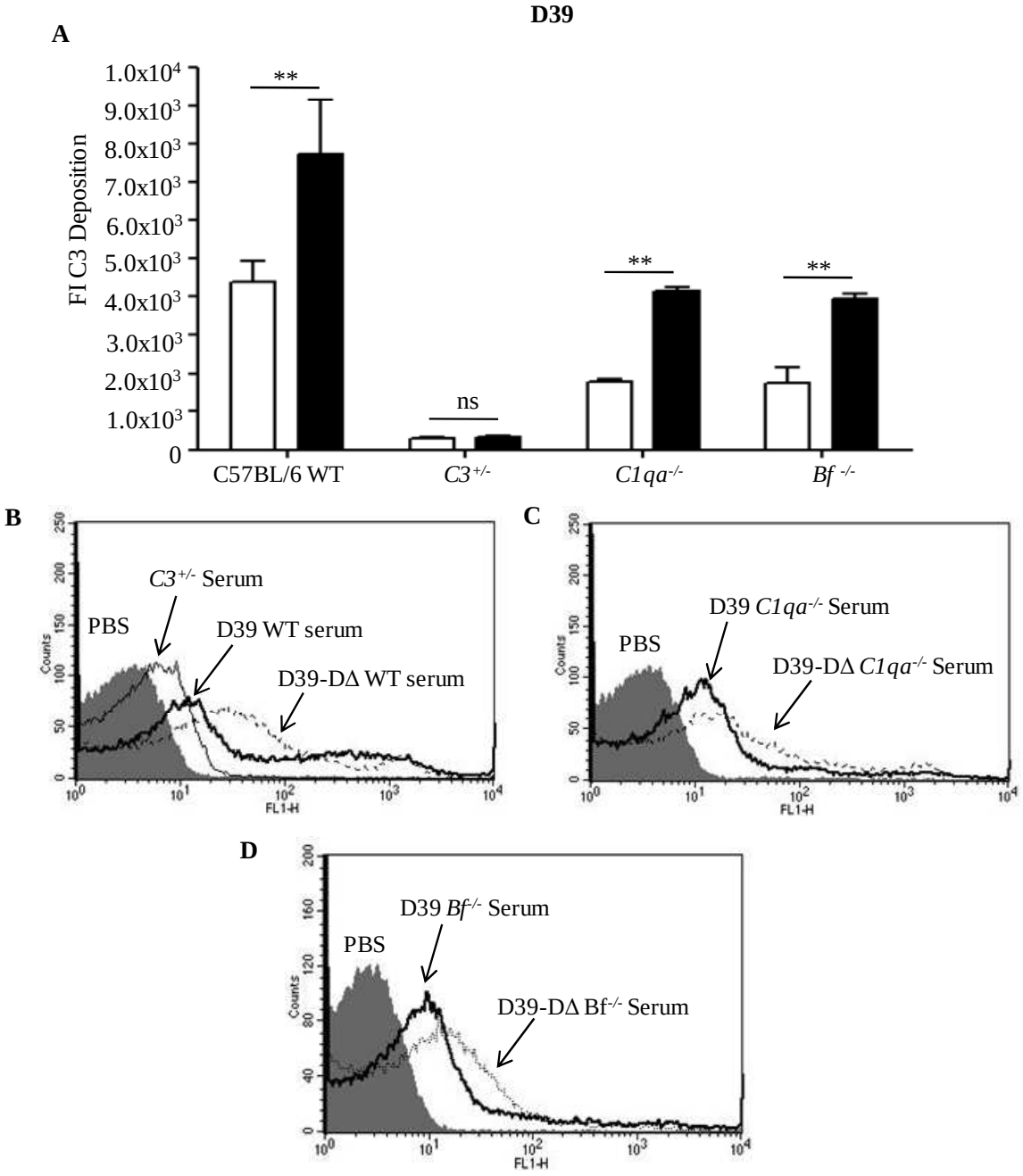


Fig 3.11 C3b/iC3b deposition on D39 strains opsonised in mouse serum

(A). C3b/iC3b deposition on D39 encapsulated (open bars) and D39-DΔ (solid bars) strains incubated in 10% serum from C57BL/6 wild-type (WT), *Bf*$^{-/-}$, *C1qa*$^{-/-}$ or *C3*$^{+/-}$ mice. Error bars represent SDs, ** $P < 0.001$ (ANOVA with post hoc tests). (B), (C), (D) Representative flow cytometry histogram of C3b/iC3b deposition on D39 strains incubated in 10% serum from C57BL/6 WT or *C3*$^{-/-}$ (B), *C1qa*$^{-/-}$ (C) and *Bf*$^{-/-}$ (D) mice.

3.2.6 The capsule affects binding of mediators of both the classical and alternative complement pathways

Both innate and adaptive immune responses use the classical pathway to initiate C3b/iC3b deposition on *S. pneumoniae* and are activated by recognition of the bacteria by specific IgG, natural IgM, CRP and SAP (Szalai et al. 1996; Brown et al. 2002; Yuste et al. 2007), whereas the alternative pathway acts mainly to amplify C3b/iC3b deposition (Walport 2001; Xu et al. 2001; Brown et al. 2002). The binding of classical pathway mediators was investigated using flow cytometry assays. Both the TIGR4*cps* and D39-DΔ strains showed increased binding of the serum proteins C1q and CRP which are known to initiate complement activity, compared to the deposition of these classical complement pathway activators on encapsulated bacteria (Fig 3.12 A and C). Additionally there was also increased IgG and natural IgM binding on the both the TIGR4*cps* and the D39-DΔ strains compared to the encapsulated parental strains (Fig. 3.13 A and C, $P < 0.001$). Both IgG and IgM are able to activate the C1-complex and hence the classical complement pathway, suggesting increased classical pathway activation through both complement factors and antibody activation. However, there was reduced binding of SAP (Fig 3.14 A), another protein that initiates classical pathway activity, to the unencapsulated bacteria for both strains. Overall these results suggest that the capsule can inhibit classical pathway activity by preventing the binding of important classical pathway mediators to the bacterial surface. In addition, there was increased factor H (FH) binding on both TIGR4*cps* and D39-DΔ compared to the parental strain, which would tend to decreased total C3 deposition on the bacterial surface by preventing alternative pathway activity (Fig 3.14 C). These effects of the capsule might be expected to decrease C3b/iC3b deposition on *S. pneumoniae*.

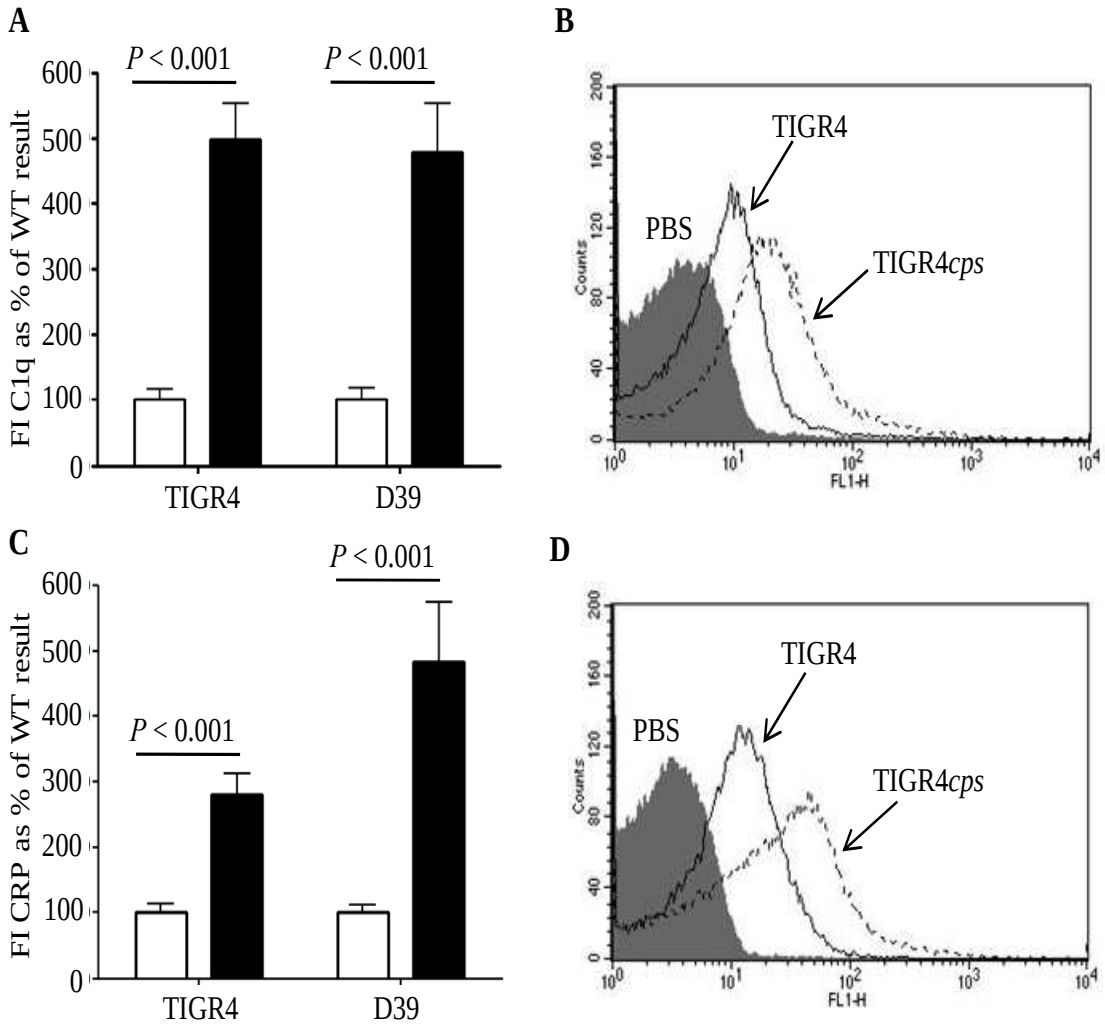
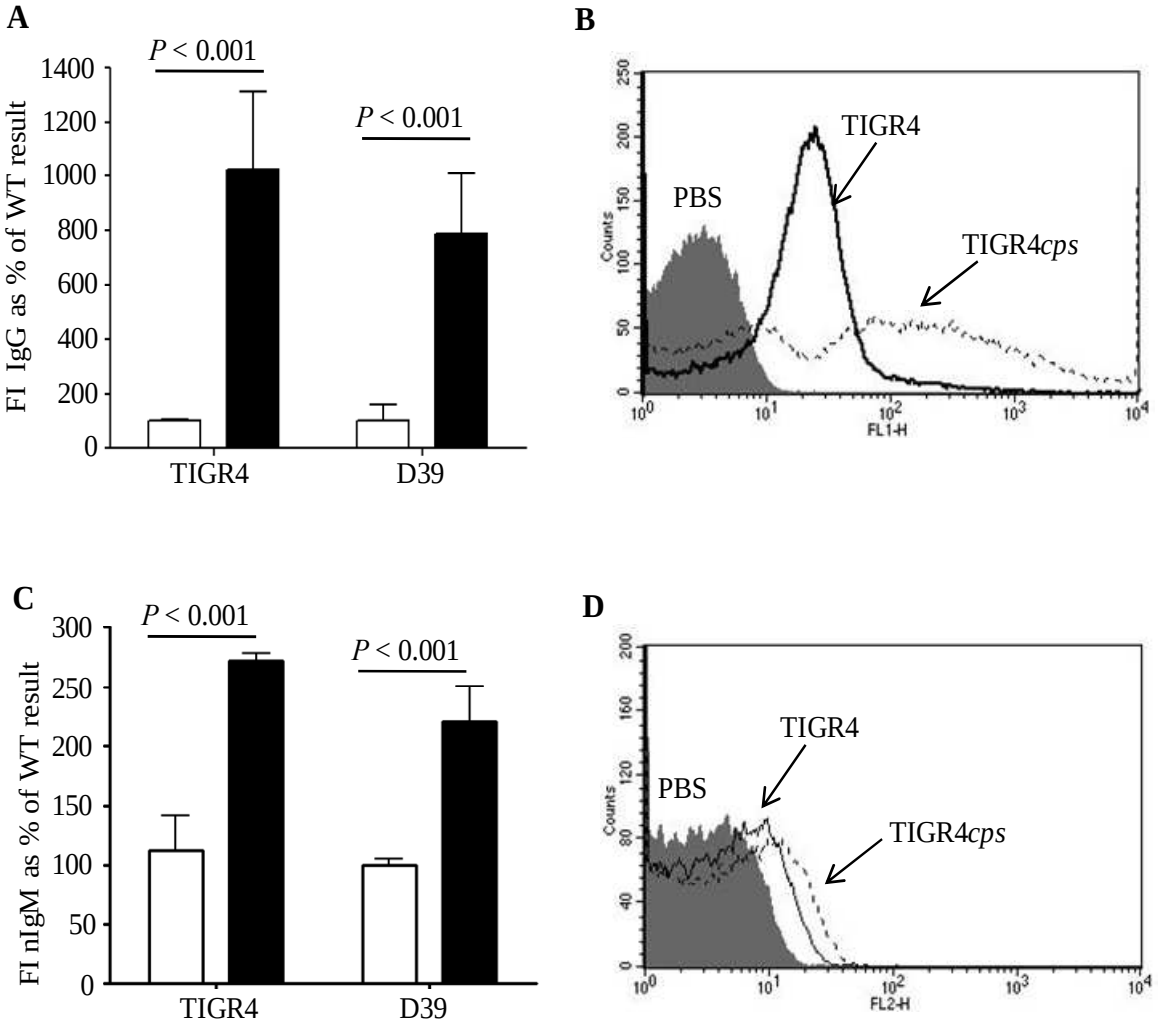


Fig 3.12 C1q and CRP binding to *S. pneumoniae* TIGR4 and D39 strains
(A), (C) FI expressed as percentage of results obtained in encapsulated strains of C1q (A) and CRP (C) on unencapsulated *S. pneumoniae* strains when compared to deposition on WT TIGR4 and D39 in 25% serum as measured by flow cytometry. Results for encapsulated strains are shown as open bars and unencapsulated as closed bars. (B), (D) Example flow cytometry histograms of C1q (B) and CRP (D) binding to TIGR4 WT (solid line) and unencapsulated (dashed line) strains in 25% serum. For all panels *P*-values represent the result of unpaired t-tests.



<u>Fig 3.13</u> IgG and natural IgM binding to *S. pneumoniae* TIGR4 and D39 strains
(A), (C) FI expressed as percentage of results obtained in encapsulated strains of IgG (A) and nIgM (C) in 25% human or mouse serum respectively, on unencapsulated strains when compared to deposition on WT TIGR4 and D39, as measured by flow cytometry. (B), (D) Results for encapsulated strains are shown as open bars and unencapsulated as closed bars. Example flow cytometry histograms of IgG (B) and nIgM (D) binding to TIGR4 WT (solid line) and TIGR4*cps* (dashed line) strains in 25% serum. For all panels *P* -values represent unpaired t-tests.

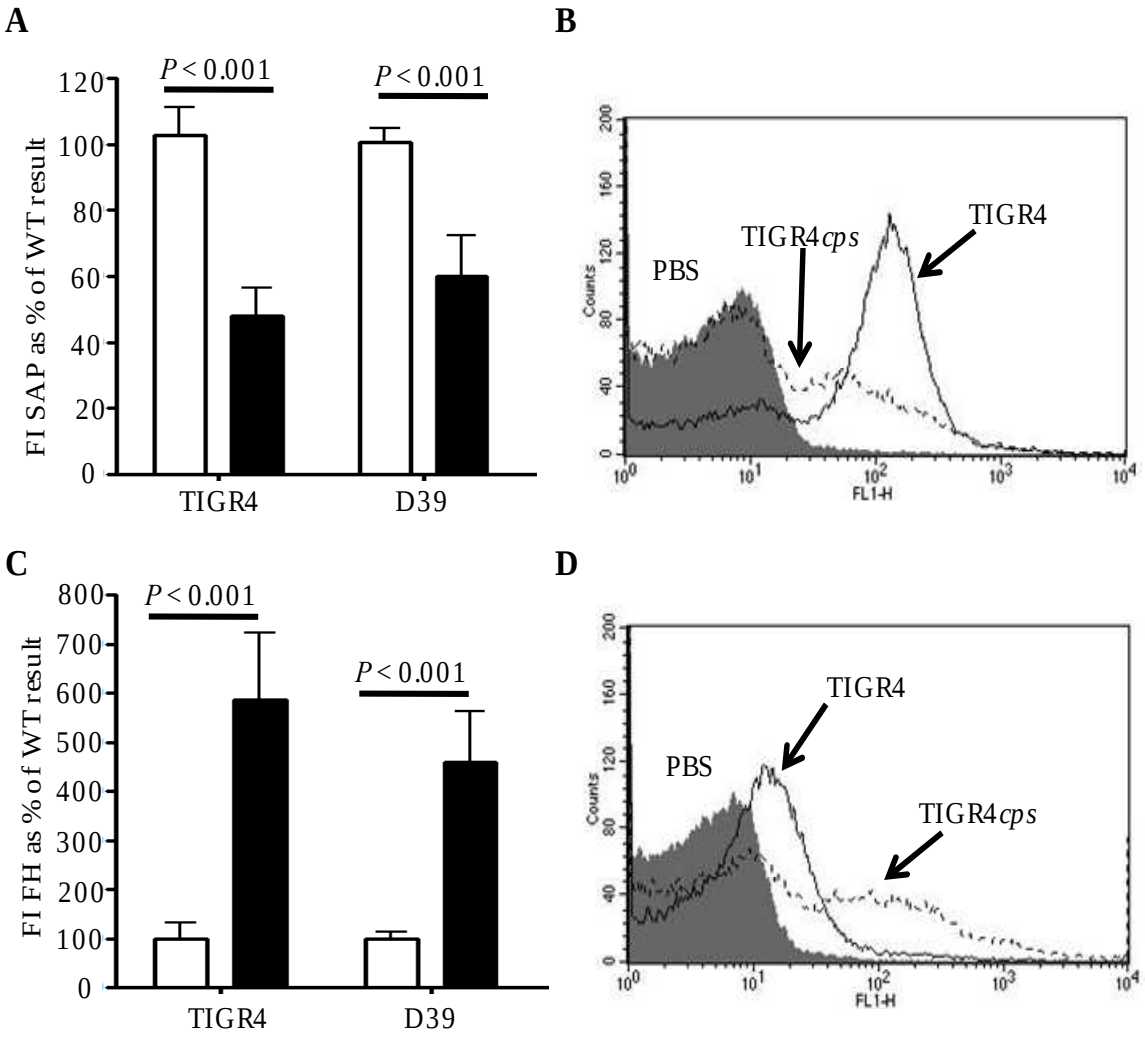


Fig 3.14 SAP and FH deposition on *S. pneumoniae* TIGR4 and D39 strains
(A), (C) FI expressed as percentage of results obtained in encapsulated strains of SAP (A), and factor H (C) on unencapsulated strains when compared to deposition on WT TIGR4 and D39 in 25% human serum, as measured by flow cytometry. Results for encapsulated strains are shown as open bars and unencapsulated as closed bars. (B), (D) Example flow cytometry histograms of SAP (B) and factor H (D) binding to TIGR4 WT (solid line) and TIGR4*cps* (dashed line) strains in 25% serum. For all panels *P* -values represent unpaired t-tests.

3.2.7 Immunogold EM C3b/iC3b deposition on *S. pneumoniae*

Immunogold EM was used to compare the distribution of C3b/iC3b deposition on encapsulated and unencapsulated *S. pneumoniae* after incubation in PBS or normal human serum. Bacteria incubated in PBS showed no immunogold staining with an anti C3b/iC3b antibody. Few gold particles were seen on the surface of the TIGR4 and D39 encapsulated strains which had been incubated in serum, and there were never more than 4 in close proximity to each other (Fig. 3.15 A and B). In contrast, on the surface of both the TIGR4*cps* and D39-DΔ strains immunogold EM for C3b/iC3b identified large numbers of gold particles, largely within one or more clusters containing a median of 29 (IQR 26-51) and 30 (IQR 15-56) gold particles for the TIGR4*cps* and D39-DΔ strains respectively ($P < 0.001$, Fig. 3.15 C, D, and E). These data support the results previously obtained with flow cytometry showing that in the absence of the polysaccharide capsule there is a marked increase in C3b/iC3b deposition on *S. pneumoniae*. Furthermore, the EM immunogold demonstrates this is mainly due to a focal accumulation at specific sites as opposed to a diffuse increase over the bacterial cell surface.

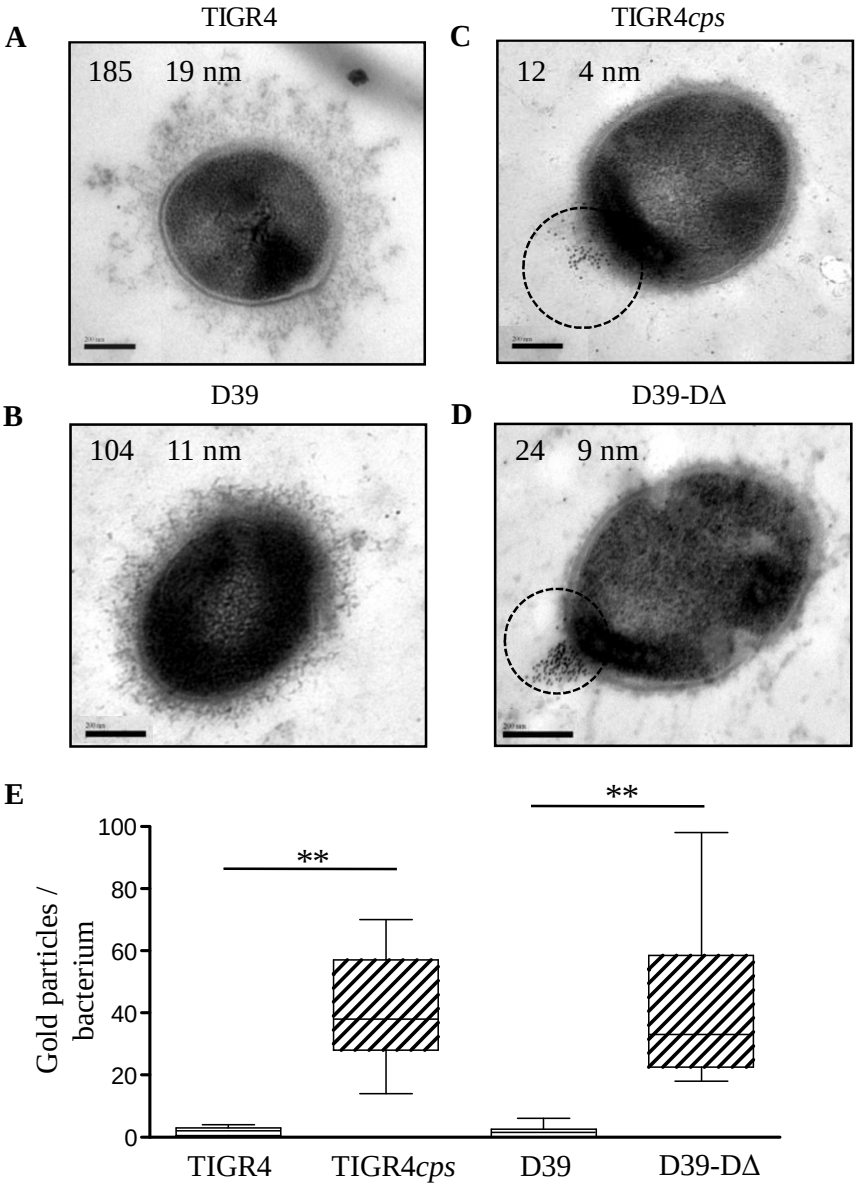


Fig 3.15 Immunogold against C3b/iC3b on TIGR4 and D39 strains

(A, B, C, D). Electron microscopy images (100,000X) of TIGR4, TIGR4*cps*, D39 and D39-DΔ strains incubated in 20% human serum and stained with immunogold against C3b/iC3b. (E). Analysis of immunogold particles on 10 random bacteria for TIGR4 and D39 encapsulated and unencapsulated strains. ** $P < 0.001$ (Kruskal-Wallis with Dunn's multiple comparison).

3.2.8 The effect of antibody on C3b/iC3b deposition

In order to characterize the relative importance of the increased IgG binding for the increased complement activity against the unencapsulated strains compared to direct inhibition of complement activity, IdeS was used to cleave the Fc fragment of the IgG in serum. IdeS treated serum showed abrogated IgG binding to TIGR4, TIGR4*cps*, D39 and D39-DΔ (Fig 3.16 A, Table 3.2). Previous results had indicated that there was an increased C1q binding to the TIGR4*cps* and D39-DΔ strains (Fig 3.12A), which is compatible with the increased binding of antibody and CRP to the unencapsulated bacteria. This increase in C1q binding persisted when serum was treated with IdeS, showing that it was partially dependent on IgG (Fig 3.16 C, Table 3.2).

For all strains C3b/iC3b deposition was reduced in IgG depleted or control sera (incubated with BSA rather than IdeS) compared to untreated sera, probably due to the breakdown of complement during the treatment process prior to the C3b/iC3b assays. In keeping with previous results, there was increased C3b/iC3b deposition on the TIGR4*cps* and D39-DΔ strains compared to the TIGR4 and D39 strains respectively in control sera (Table 3.2). IgG depletion reduced C3b/iC3b deposition on the TIGR4*cps* and D39-DΔ strains, demonstrating that complement deposition on unencapsulated strains is partially dependent on IgG (Table 3.2). However, increased C3b/iC3b deposition persisted on unencapsulated strains compared to encapsulated strains showing the effect of the capsule has a significant IgG-independent component.

Table 3.2 Effects of IgG depletion using IdeS on mean FI +/- SDs of C3b/iC3b deposition and C1q binding to unencapsulated (-cps) and encapsulated (+cps) D39 and TIGR4 strains in 50% human serum. *P* values represent comparisons between the results for unencapsulated and encapsulated strains using unpaired Student's t tests.

Assay	Strain	IdeS treated	+cps	-cps	*P* value
IgG Binding	D39	No	1575 ± 100	2810 ± 200	< 0.001
		Yes	140 ± 18	131 ± 16	< 0.001
	TIGR4	No	1476 ± 180	2698 ± 202	< 0.001
		Yes	123 ± 16	135 ± 17	< 0.001
C3b/iC3b deposition	D39	No	12118 ± 2200	28776 ± 3900	< 0.001
		Yes	9466 ± 440	19856 ± 4400	< 0.001
	TIGR4	No	20385 ± 2600	38056 ± 3400	< 0.001
		Yes	12133 ± 1900	20978 ± 1900	< 0.001
C1q binding	D39	No	1073 ± 110	3524 ± 190	< 0.001
		Yes	719 ± 150	1357 ± 100	< 0.001
	TIGR4	No	1041 ± 210	4520 ± 200	< 0.0001
		Yes	933 ± 110	1648 ± 210	< 0.001

3.2.9 The effect of *S. pneumoniae* capsule on complement-dependent and -independent neutrophil phagocytosis

In order to examine the functional consequences of the increased C3b/iC3b deposition on unencapsulated *S. pneumoniae* strains, an established flow cytometry assay of neutrophil phagocytosis was used (Yuste et al. 2007; Yuste et al. 2008). Neutrophils were extracted by Ficoll-dextran sedimentation from blood obtained from healthy volunteers, and experiments were replicated using neutrophils from at least 3 different donors. Fluorescently labelled *S. pneumoniae* strains were opsonised in either HBSS, 20% serum in which complement had been inactivated by heat treatment (Fig 2.1), or 5%, 10% and 20% normal human serum before mixing with neutrophils. As previously shown, the association of bacteria with neutrophils (assessed by flow cytometry) was increased in strains which were opsonised in normal serum compared to heat treated serum (Fig 3.16 and 3.17) (Yuste et al. 2008). Additionally there was a dose response to increasing concentrations of human serum in all strains. Compared to the encapsulated strains there were higher levels of association with human neutrophils of both the TIGR4*cps* and D39-DΔ strains in all the concentrations of serum, showing the capsule inhibits complement-dependent bacterial association with neutrophils. In addition there was an increased association of the TIGR4*cps* and D39-DΔ strains compared to their parental strains in heat treated serum, in which complement is inactivated, suggesting there is also a complement-independent capsule effect.

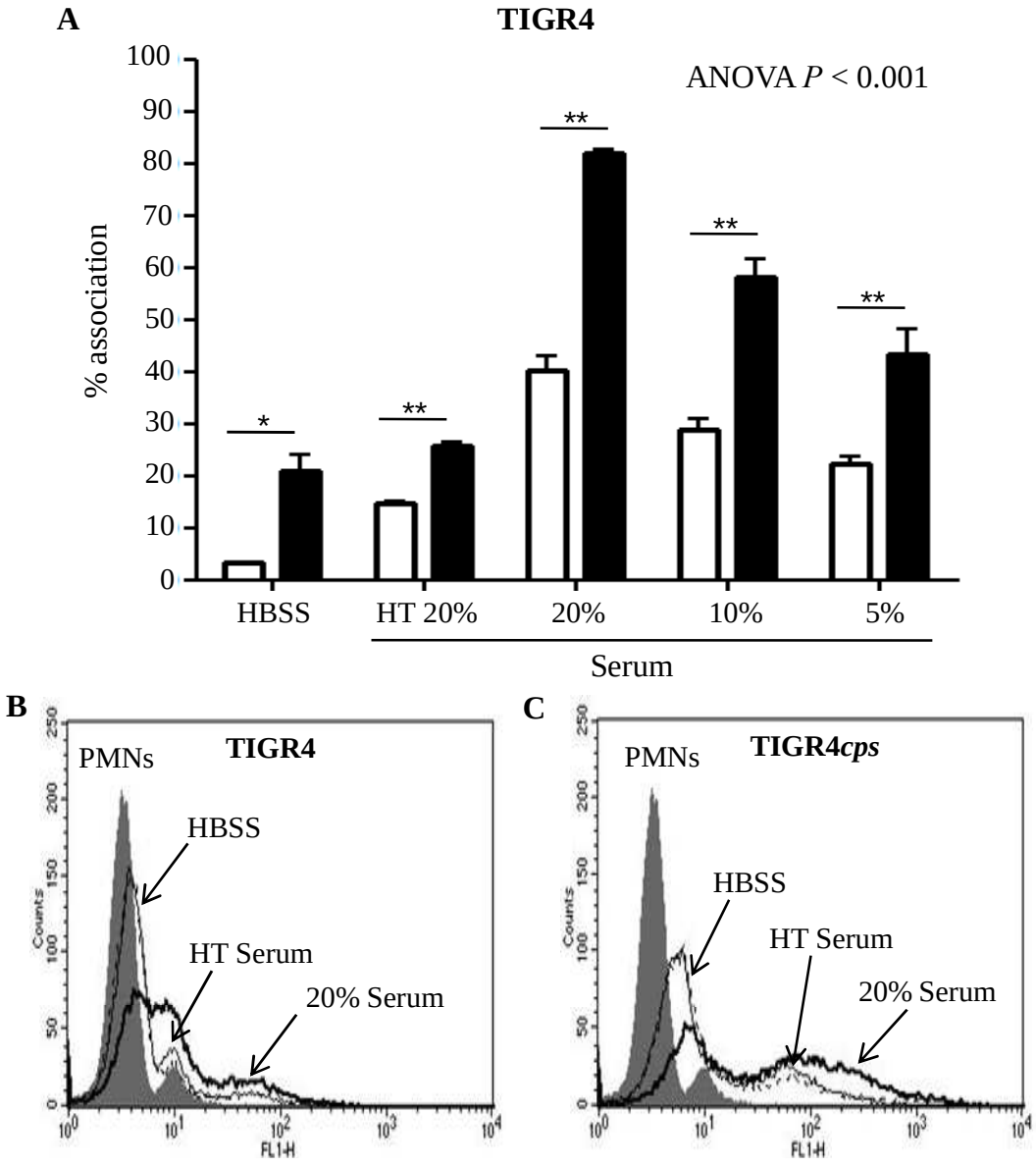
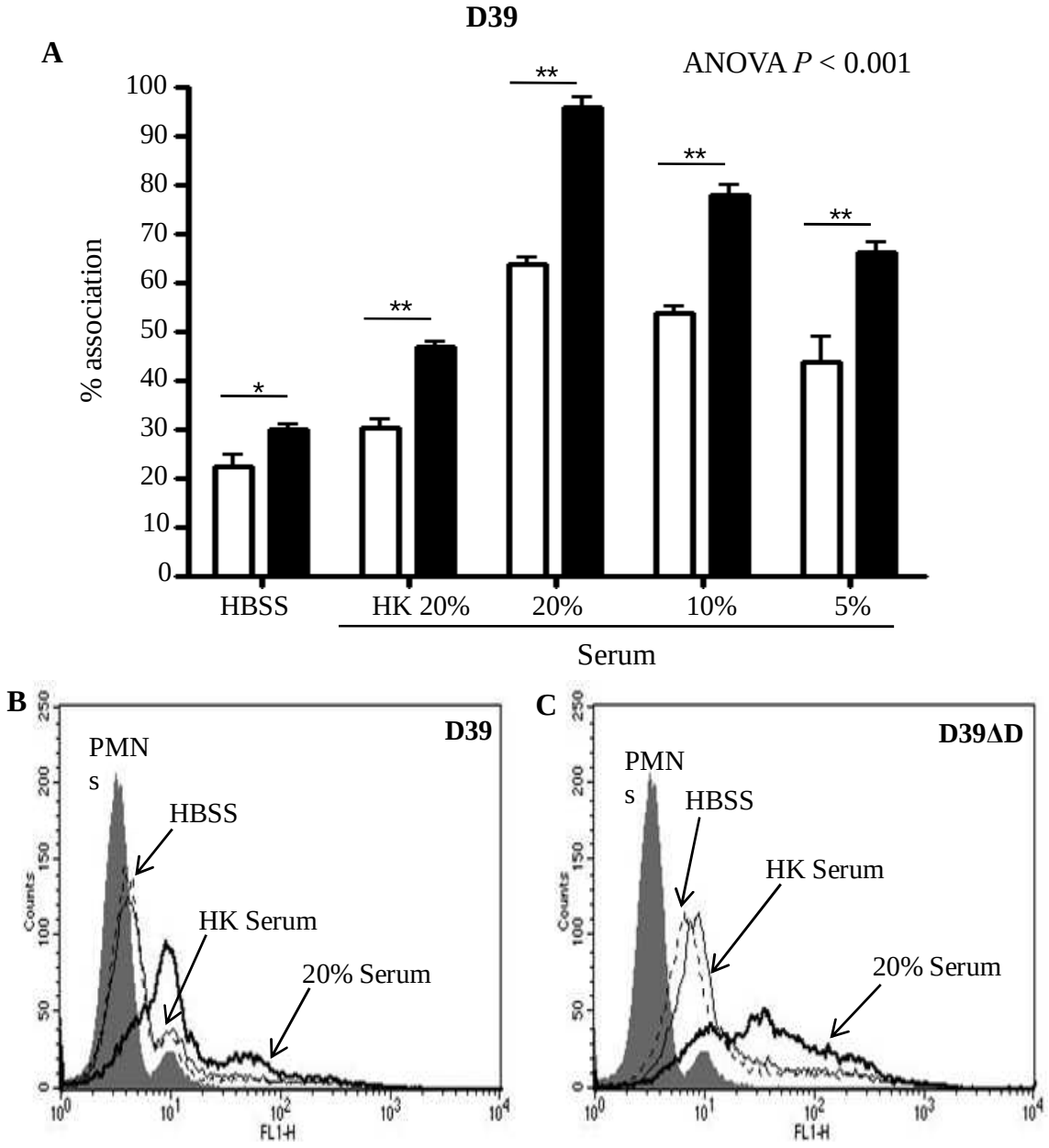


Fig 3.16 Neutrophil phagocytosis of TIGR4 strains opsonised in human serum
(A). Percentage of neutrophils associated with TIGR4 (open bars) or TIGR4*cps* (closed bars) *S. pneumoniae* strains incubated in different opsonins, as determined by flow cytometry. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests). (B), (C) Representative example flow cytometry histograms for neutrophil association with TIGR4 (B) and TIGR4*cps* (C) strains when incubated in HBSS (dashed line), heat inactivated 20% serum (thin solid line) and 20% human serum (thick solid line).



<u>Fig 3.17</u> Neutrophil phagocytosis of D39 strains opsonised in human serum
(A). Percentage of neutrophils associated with D39 (open bars) or D39-DΔ (closed bars) *S. pneumoniae* strains incubated in different opsonins, as determined by flow cytometry. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests). (B), (C) Representative example flow cytometry histograms for neutrophil association with D39 (B) and D39-DΔ (C) D39 strains when incubated in HBSS (dashed line), heat inactivated 20% serum (thin solid line) and 20% human serum (thick solid line).

In order to determine the effect of the capsule on the proportion of *S. pneumoniae* bacteria that were cell-surface associated compared to phagocytosed by the neutrophils, the assays were repeated in 20% serum using Trypan Blue to quench fluorescence from bacteria on the cell surface but not internalised, and/or 1μM cytochalasin D to inhibit actin polymerisation and therefore phagocytosis. For reactions treated with Trypan Blue there were similar increases in the association of the TIGR4*cps* and D39-DΔ strains compared to the TIGR4 and D39 strains as found for the untreated reactions. In contrast, there were no increases in the association of the TIGR4*cps* and D39-DΔ strains with neutrophils compared to results obtained with the TIGR4 and D39 strains when neutrophils were pre-treated with cytochalasin (Fig 3.18 and 3.19). These data confirm that the majority of the *S. pneumoniae* capsule effect on association with neutrophils is through inhibition of phagocytosis rather than by reducing association of the bacteria with the neutrophil surface.

To further examine complement-dependent and –independent effects on neutrophil association, the assays were repeated using the human serum deficient in the single complement components C3, C1q, Bf and C9. There were significant differences in neutrophil association between the unencapsulated and encapsulated strains in all the conditions tested, including for bacteria incubated in $C3^-$ serum or HBSS alone (Fig 3.20 A and B). Experiments using serum from wild-type, $C3^{+/-}$, $C1qa^{-/-}$ or $Bf^{/-}$ C57BL/6 mice gave similar results to those obtained with depleted human serum (Fig 3.21 A and B). These results demonstrated that for both the TIGR4 and D39 strains the capsule inhibits neutrophil association by classical and alternative pathway mediated complement-dependent mechanisms as well as by complement–independent mechanisms.

Depletion of IgG from the sera reduced neutrophil phagocytosis of all strains in both normal and $C3^-$ sera, confirming the importance of IgG for *S. pneumoniae* phagocytosis (Table 3.3). However, there was a persisting increase in phagocytosis of unencapsulated compared to encapsulated strains after incubation in IdeS treated normal and $C3^-$ sera, demonstrating that the capsule has effects on phagocytosis even in the absence of IgG alone and in the absence of both complement and IgG respectively (Table 3.3).

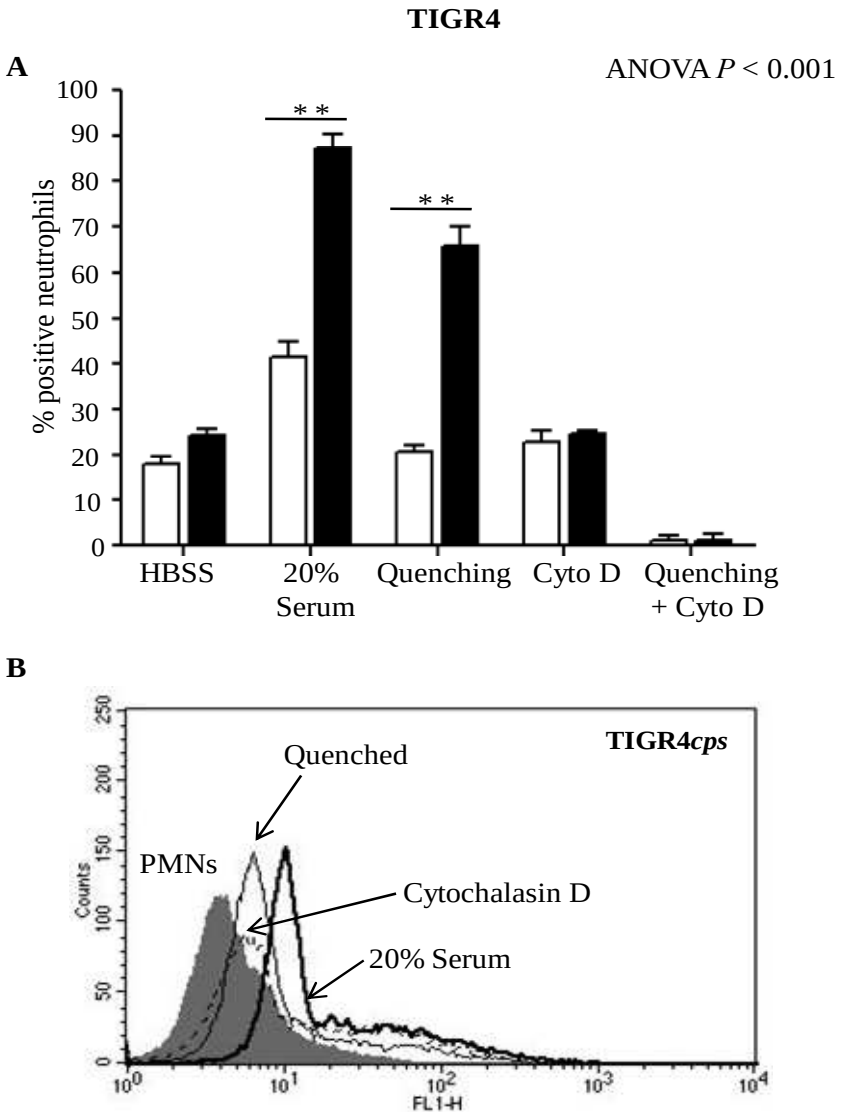


Fig 3.18 Effect of cytochalasin D on TIGR4 strain association with neutrophils
(A). Percentage of FL-1 positive neutrophils when incubated with TIGR4 (open bars) or TIGR4*cps* (closed bars) *S. pneumoniae* strains, as determined by flow cytometry. Neutrophils were untreated and bacteria opsonised with HBSS or 20% serum, or neutrophils were treated with 1μM cytochalasin D (inhibiting internalisation of bacteria) or the FAM-SE signal was quenched using Trypan Blue (results representing internalised bacteria). Cells treated with both cytochalasin D and Trypan B returned to background fluorescence. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests). (B). Representative example flow cytometry histograms for neutrophil association with TIGR4*cps* when incubated with cytochalasin treated cells (dashed line) or when the FAM-SE signal was quenched with Trypan Blue (thin solid line) and 20% human serum (thick solid line) incubation alone.

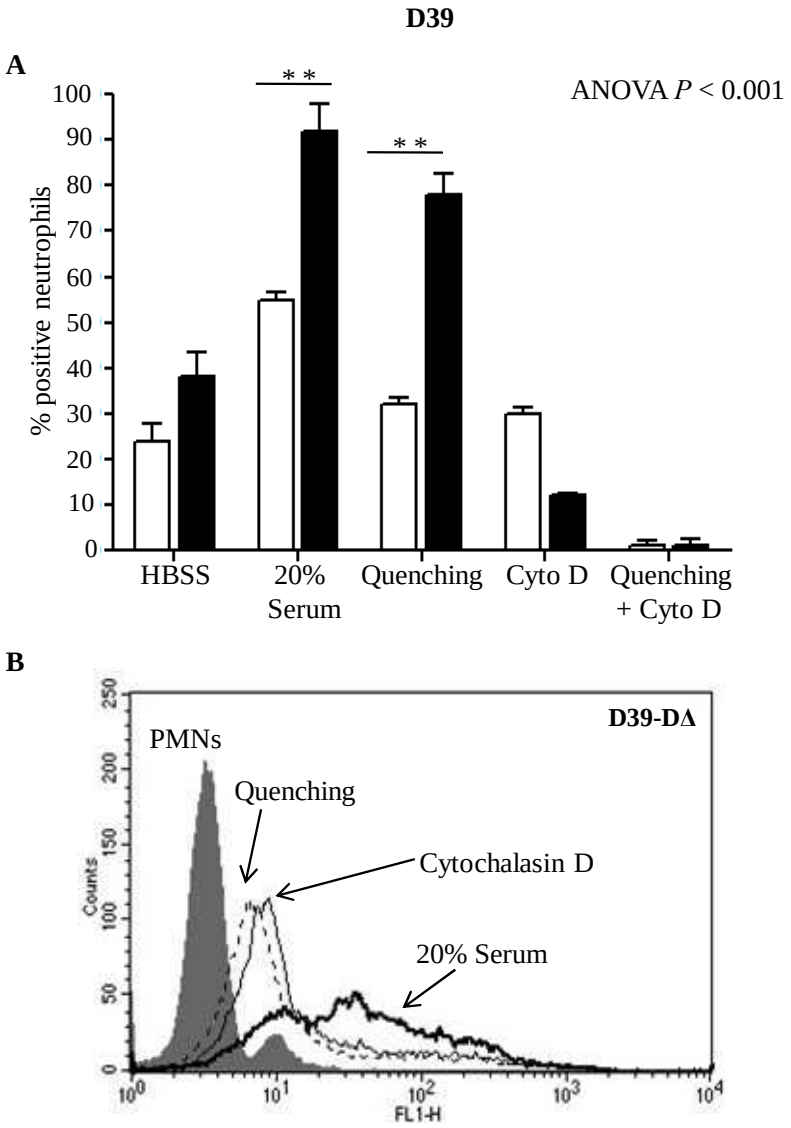


Fig 3.19 Effect of cytochalasin D on D39 strain association with neutrophils

(A). Percentage of FL-1 positive neutrophils when incubated with D39 (open bars) or D39-DΔ (closed bars) *S. pneumoniae* strains, as determined by flow cytometry. Neutrophils were untreated and bacteria opsonised with HBSS or 20% serum, or neutrophils were treated with 1μM cytochalasin D (inhibiting internalisation of bacteria) or the FAM-SE signal was quenched using Trypan Blue (results representing internalised bacteria). Cells treated with both cytochalasin D and Trypan B returned to background fluorescence. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests). (B). Representative example flow cytometry histograms for neutrophil association with D39-DΔ when incubated with cytochalasin treated cells (dashed line) or when the FAM-SE signal was quenched with Trypan Blue (thin solid line) and 20% human serum (thick solid line) incubation alone.

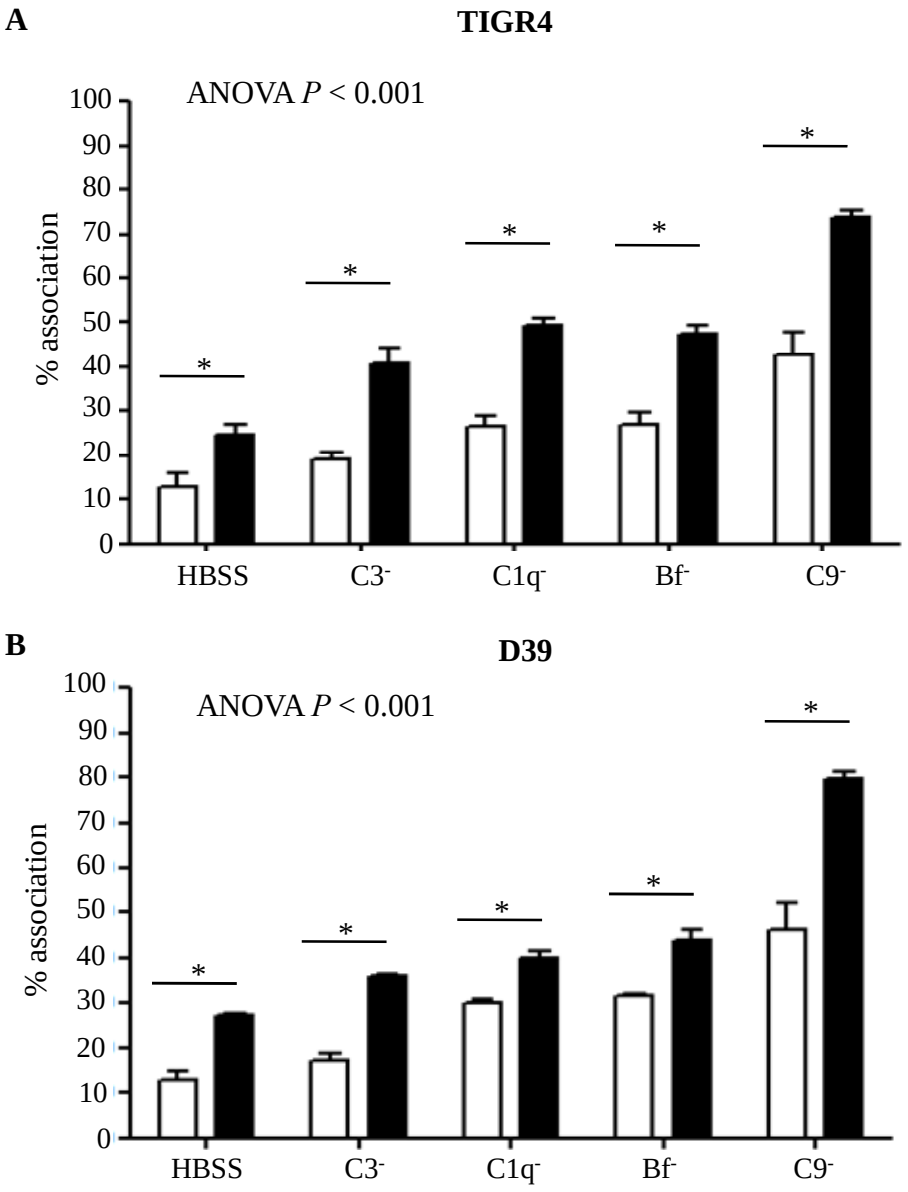


Fig 3.20 Effect of depletion of specific complement factors on phagocytosis of encapsulated and unencapsulated strains

(A), (B) Percentage of neutrophils associated with encapsulated (open bars) or unencapsulated (solid bars) *S. pneumoniae* TIGR4 (A) or D39 (B) strains when opsonised in HBSS or 10% human serum depleted of C3, C1q, Bf or C9. For both panels, error bars represent SDs, * $P < 0.001$ (ANOVA with post-hoc tests).

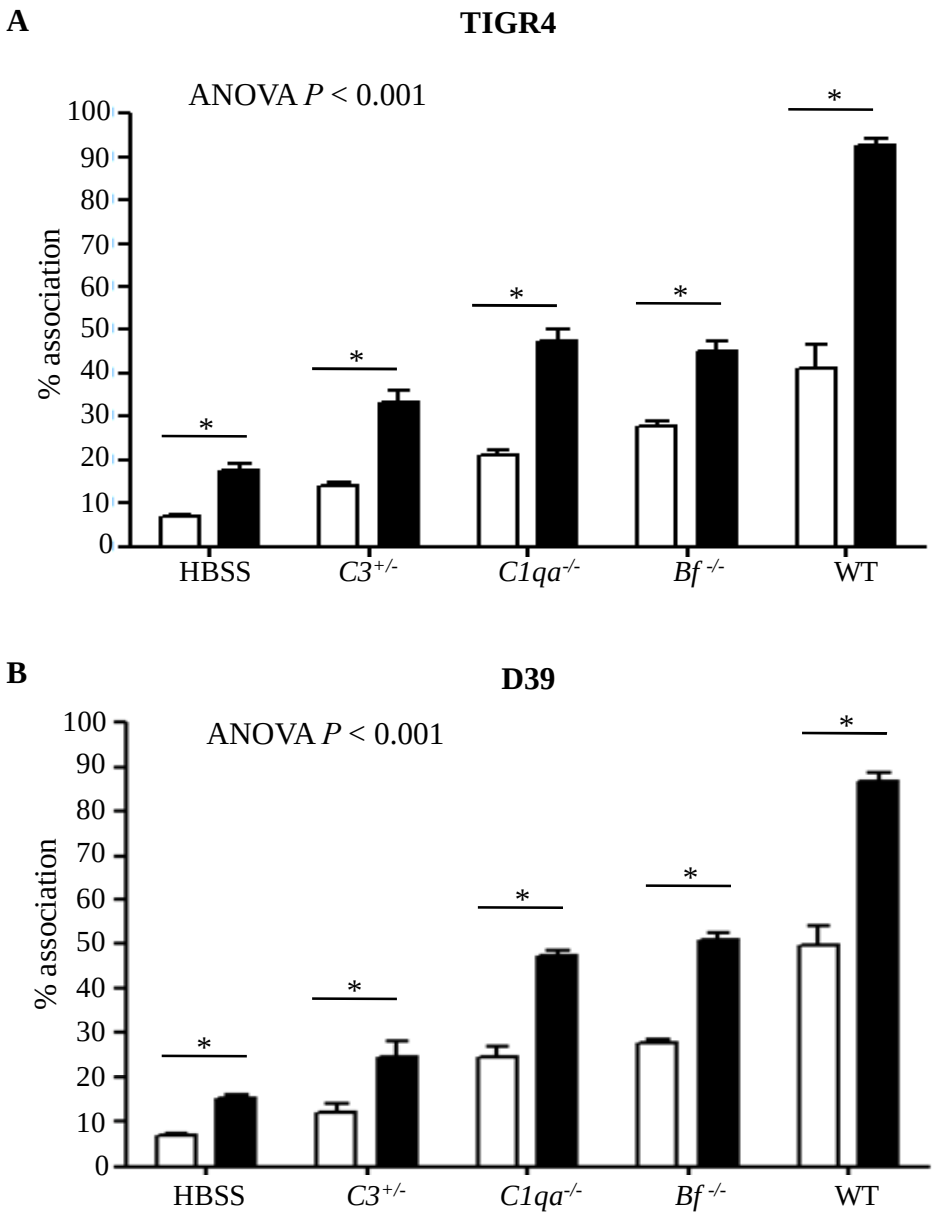


Fig 3.21 Phagocytosis of encapsulated and unencapsulated strains in serum from complement deficient mice

(A), (B) Percentage of neutrophils associated with encapsulated (open bars) or unencapsulated (solid bars) *S. pneumoniae* TIGR4 (A) or D39 (B) strains when opsonised in HBSS or 20% C57BL/6 mouse serum from $C3^{+/-}$, $C1qa^{-/-}$, $Bf^{-/-}$ or WT. For both panels, error bars represent SDs, * $P < 0.001$ (ANOVA with post-hoc tests).

Table 3.3 Effects of IgG depletion using IdeS on mean +/- SDs percentage association of unencapsulated (-cps) and encapsulated (+cps) D39 and TIGR4 strains with neutrophils after incubation in 20% normal human serum (NHS) or 20% C3 deficient serum ($C3^-$). *P* values represent comparisons between the results for unencapsulated and encapsulated strains using unpaired Student's t tests.

Serum	Strain	IdeS treated	+cps	-cps	*P* value
NHS	D39	No	39.2 ± 2.5	79.0 ± 5.6	< 0.0001
		Yes	28.5 ± 0.5	61.0 ± 2.8	< 0.0001
	TIGR4	No	32.0 ± 3.9	85.9 ± 3.9	< 0.0001
		Yes	28.9 ± 1.5	70.2 ± 1.2	< 0.0001
$C3^-$	D39	No	23.5 ± 0.8	39.9 ± 1.3	< 0.0001
		Yes	16.8 ± 1.1	25.8 ± 1.2	< 0.0001
	TIGR4	No	21.2 ± 3.2	40.2 ± 2.7	< 0.0001
		Yes	12.8 ± 0.8	28.5 ± 1.1	< 0.0001

3.2.10 Complement deficiency partially restores virulence in a mouse model of septicaemia

Virulence of the unencapsulated *S. pneumoniae* strains was investigated in a mouse model of sepsis. In this model, bacteria are inoculated IP and even very low doses (down to 10^1 CFU) of wild-type TIGR4 or D39 bacteria rapidly cause septicaemia which is usually fatal within 48 hours (Brown et al. 2002; Morona et al. 2004). However, in keeping with previous reports (Wood et al. 1949; Morona et al. 2004) TIGR4*cps* and D39-DΔ strains were not recoverable from the spleens or blood of mice 24 hours after inoculation IP with 5 x 10^3 CFU, whereas mice inoculated with the TIGR4 or D39 strains had over 6 $\log_{10}$ CFU in spleen homogenates (Table 3.4). However, when $C3^{+/-}$ mice were inoculated IP with the TIGR4*cps* and D39-DΔ strains, within 24 hours over 5 $\log_{10}$ CFU of bacteria were present in spleen homogenates and blood (Table 3.4)**,** demonstrating that the virulence of the unencapsulated bacteria was restored with decreased C3 levels. However, complement deficient mice are highly susceptible to *S. pneumoniae* (Wood et al. 1949; Brown et al. 2002) and the increase in virulence of the unencapsulated strains in these mice does not necessarily mean that the effect of the capsule in this model is mediated through effects on complement-dependent immunity. Hence, to directly compare the effects of complement on the comparative virulence of the TIGR4*cps* and D39-DΔ strains to the TIGR4 and D39 strains, mixed infection experiments in wild-type and complement deficient mice were performed. Wild-type, $C3^{+/-}$, $C1qa^{-/-}$ and $Bf^{-/-}$ mice were inoculated with a 50/50 ratio of TIGR4*cps* and TIGR4 bacteria or D39-DΔ and D39 bacteria, and the ratio of each strain amongst bacteria recovered from mouse spleens or blood 24 hours after inoculation identified by plating on plain and antibiotic-containing medium and used to calculate a competitive index (CI). As expected no TIGR4*cps* or D39-DΔ bacteria were recovered

from either the spleen or the blood of wild-type C57BL/6 mice whereas greater than $\log_{10}$ 6 ml^{-1} bacterial CFU of the TIGR4 and D39 strains were found in the blood or spleen homogenates, giving CIs which were all less than $\log_{10}$ -5.1 (Fig. 3.23 and 3.24). In contrast in *C3*$^{+/-}$ mice a significant proportion of bacteria recovered from the spleen or blood were the unencapsulated strain, giving a CI of $\log_{10}$ -3.1 (IQR -3.3 to -3.0) in blood and -2.2 (IQR -3.0 to -1.9) in spleens for the TIGR4*cps* strain and $\log_{10}$ -3.0 (IQR -3.1 to -2.9) in blood and -2.1 (IQR -3.0 to -2.0) in spleen for the D39-DΔ strain. Mixed infection experiments in *C1qa*$^{-/-}$ and *Bf*$^{-/-}$ mice also showed a lesser but similar order of magnitude increase in CI compared to wild-type mice (Fig. 3.23 and 3.24). These data demonstrate that the effect of the capsule on *S. pneumoniae* virulence in this model is dependent on complement and is mediated by both the alternative and classical pathways. However, even in *C3*$^{+/-}$ mice the unencapsulated strains were still markedly attenuated in virulence, with only approximately 1 in 1000 bacteria present in the blood and less than 1 in bacteria 100 recovered from the spleen being the TIGR4*cps* or the D39-DΔ strain rather than the corresponding encapsulated strain. Hence there is also likely to be a major complement-independent effect of the capsule on the virulence of *S. pneumoniae* in the sepsis model.

Table 3.4 Log_{10} bacterial CFU recovered from splenic homogenates and blood 24 hours after IP inoculation of wild-type (C57B/6) or *C3*$^{+/-}$ mice with 5000 CFU of wild type or unencapsulated *S. pneumoniae* strains.

Bacterial strain	Mouse strain	Mean (SD) log_{10} CFU (n = 4 to 5) Spleen	Mean (SD) log_{10} CFU (n = 4 to 5) Blood
TIGR4	C57B/6	6.55 (0.32)	6.73 (0.10)
TIGR4*cps*	C57B/6	0 (0)	0 (0)
TIGR4*cps*	*C3*$^{+/-}$	5.08 (0.03)	5.03 (0.11)
D39	C57B/6	6.84 (0.06)	6.58 (0.78)
D39-DΔ	C57B/6	0 (0)	0 (0)
D39-DΔ	*C3*$^{+/-}$	5.10 (0.06)	5.21 (0.13)

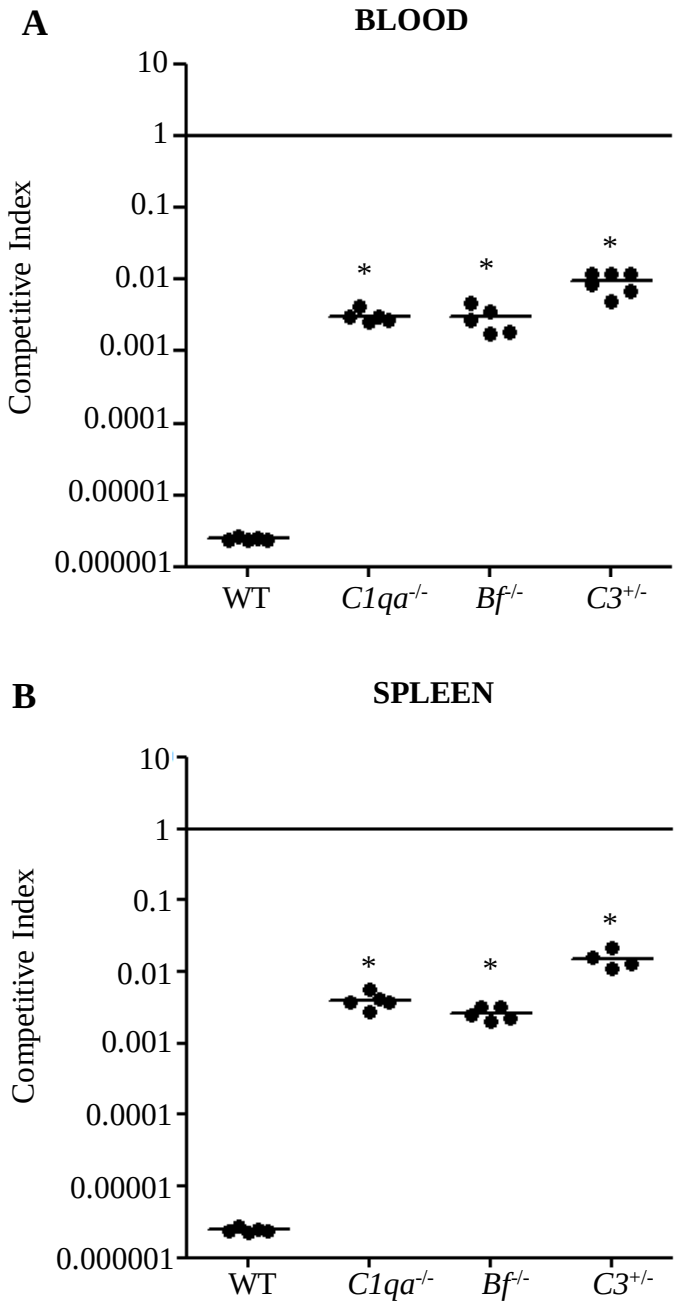


Fig 3.22 TIGR4 mixed infection experiments in complement deficient mice
(A), (B) CIs for mixed infections with the TIGR4 and TIGR4*cps* strains expressed as $\log_{10}$ for bacteria recovered from mouse blood (A) or spleens (B) after intraperitoneal inoculation with 5000 CFU in total. Mouse strain is given on the X axis, and for the results obtained for complement deficient mice versus those obtained for wild-type mice * P=0.001 (Kruskal-Wallis with post hoc analysis).

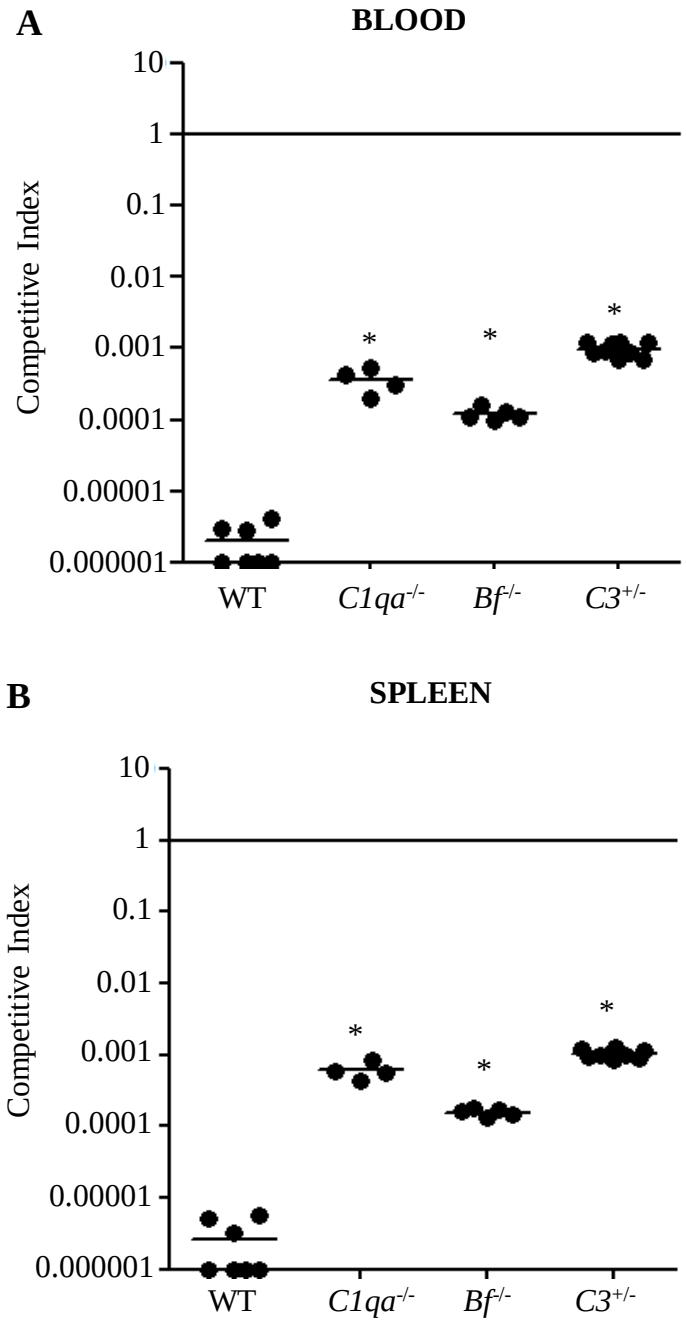


Fig 3.23 D39 mixed infection experiments in complement deficient mice
(A) and (B) CIs for mixed infections with the D39 and D39-DΔ strains expressed as $\log_{10}$ for bacteria recovered from mouse blood (A) or spleens (B) after intraperitoneal inoculation with 5000 CFU in total. Mouse strain is given on the X axis, and for the results obtained for complement deficient mice versus those obtained for wild-type mice * P=0.001 (Kruskal-Wallis with post hoc analysis).

3.3 SUMMARY

The experiments in this chapter have examined the consequences of loss of the capsule on interactions with complement and neutrophils for two *S. pneumoniae* strains, TIGR4 and D39, both of which have been used extensively for pathogenesis studies and are capable of causing severe infections in mice. Flow cytometry has confirmed the results of other investigators, showing that the capsule inhibits opsonisation of *S. pneumoniae* with C3b/iC3b for both strains, and immunoblots for C3 also show that this is associated with reduced activation of complement in sera. Data obtained with both human and mouse sera depleted in either the first component of the classical pathway C1q or the alternative pathway protein factor B demonstrated that both pathways are required for the increase in C3b/iC3b deposition on unencapsulated *S. pneumoniae*. The EM immunogold experiments showed large clusters of C3 particles at one site on the cell wall suggestive of focal amplification of complement activity only in unencapsulated stains. Taken together these results suggest that the capsule can prevent both recognition of *S. pneumoniae* by the classical pathway mediators IgG and CRP and amplification of C3b/iC3b deposition by the alternative pathway.

Neutrophil phagocytosis is considered one of the major elements of immunity to *S. pneumoniae*, and there were significant increases in phagocytosis of both of the unencapsulated strains compared to the corresponding encapsulated strains at all concentrations of complement used and in the commercially available C9, C1q and factor B depleted sera. Experiments with cytochalasin D demonstrated that the differences between unencapsulated and encapsulated bacteria were largely due to increased internalisation of unencapsulated bacteria. However as well as complement-dependent effects of the capsule

on phagocytosis, there were also significant impairments of the association of encapsulated TIGR4 and D39 with neutrophils when the bacteria were incubated in HBSS, heat treated or commercially available C3 depleted serum, all conditions in which bacteria are not opsonised with complement. These results show that as well as inhibiting neutrophil phagocytosis by reducing opsonisation of *S. pneumoniae* with C3b/iC3b, the capsule also prevents complement-independent mechanisms of phagocytosis.

Interestingly depletion of IgG from the test serum resulted in reduced C3b/iC3b deposition on the TIGR4*cps* and the D39-DΔ strains, demonstrating that complement deposition on unencapsulated strains is partially dependent on IgG. However, there was a persisting increase in C3b/iC3b deposition on unencapsulated strains compared to the encapsulated strains in IdeS treated serum. In addition, in IdeS treated serum although C1q binding to *S. pneumoniae* was reduced, there were still significant increases in C1q binding to unencapsulated compared to encapsulated strains. Furthermore, depletion of IgG from the sera reduced neutrophil phagocytosis of all strains in both normal and $C3^-$ sera. However, there was a persisting increase in phagocytosis of unencapsulated compared to encapsulated strains after incubation in IdeS treated normal and $C3^-$ sera, demonstrating that the capsule has effects on phagocytosis even in the absence of IgG alone and in the absence of both complement and IgG respectively.

The importance of the effects of the capsule on complement activity during infection was investigated using a mouse model of sepsis and genetically modified mice with reduced C3 levels, or deficient in C1q (no classical pathway activity) or factor B (no alternative pathway activity). In wild-type mice although both the D39 and TIGR4 strains are highly

virulent, even inoculation with large numbers of unencapsulated bacteria did not cause disease. In contrast, infection of *C3*$^{+/-}$ mice with unencapsulated bacteria resulted in significant septicaemia, suggesting that complement activity is required for the effects of the capsule on virulence. This was confirmed using competitive infection experiments, which showed that the unencapsulated strains were avirulent in complement sufficient mice but regained virulence when there was partial deficiency of C3 and absence C1q or factor B.

CHAPTER 4

ROLE OF CAPSULE IN INTERACTIONS WITH MACROPHAGES AND INNATE IMMUNE ACTIVATION

4.1 INTRODUCTION

A central component of early pulmonary immunity is the alveolar macrophage (AM), resident phagocytic cells found within alveoli and the bronchial tree. AMs are the first immune effector cell to interact with invading pathogens (Gordon et al. 2002) and are likely to be vital in preventing the development of significant lung disease. Depletion of AMs increases *S. pneumoniae* replication in the lung (Dockrell et al. 2003), but it remains unclear how the capsule aids development of *S. pneumoniae* respiratory infections, and if any capsular effect is complement dependent or independent. Although complement factors are present in BALF and increase during *S. pneumoniae* infection, the contribution of complement to the interactions of *S. pneumoniae* with AMs during the development of pneumonia is also unclear. Complement levels in BALF are only approximately 10% of those in serum (Gross et al. 1978), and macrophage phagocytosis of *S. pneumoniae* is slower than that of neutrophils, with significant associations taking up to 2 hours rather than 30 minutes (Gordon et al. 2000; Arredouani et al. 2004).

However both the *S. pneumoniae* capsule and complement could also aid host defense to early *S. pneumoniae* infection by effects on the inflammatory response. The role of inflammation during immunity to *S. pneumoniae* pneumonia is complex, with deficiencies of pro-inflammatory cytokines or intracellular signaling molecules resulting in poor control of bacterial replication (Nakasone et al. 2007; Sun et al. 2007), but down-regulation of

inflammatory responses by macrophages improves host survival, possibly by preventing damage to the lung and impaired gas exchange (Knapp et al. 2003). As well as promoting phagocytosis, increased activation of complement by unencapsulated *S. pneumoniae* could affect inflammatory responses. The small soluble products of C3 cleavage (C3a) is pro-inflammatory, causing vasodilation, white cell chemotaxis, impairment of neutrophil apoptosis, and the induction of cytokine production (Walport 2001). As well as the effects of C3a, bacteria opsonised by C3b and iC3b could induce inflammation indirectly by activating AMs, an important source of pro-inflammatory cytokines. Hence complement activity could aid immunity to *S. pneumoniae* pneumonia by increasing recruitment and activation of white cells directly or through the increased release of pro-inflammatory cytokines and chemokines.

In this chapter I have investigated the effect of capsule on phagocytosis of *S. pneumoniae* and a mouse macrophage cell line (RAW 264.7 cells) and alveolar macrophages in a model of early lung infection *in vivo*. In addition, I have investigated the effect of capsule in modulating the inflammatory response produced by macrophages through analyzing TNFα production both *in vitro* and *in vivo*, as well as looking a innate cell activation pathways.

4.2 RESULTS

4.2.1 The effect of *S. pneumoniae* capsule on complement-dependent and -independent phagocytosis by macrophages

In order to determine if the *S. pneumoniae* capsule had an effect on macrophage phagocytosis a flow cytometry opsonophagocytosis assay was used. Adherent RAW 264.7 cells were incubated with FAM-SE labelled *S. pneumoniae* strains which were previously opsonised in either HBSS, 20% serum in which complement had been inactivated by heat treatment, or 20% normal human serum. Within 15 minutes, there was a significant complement dependent association with RAW 264.7 cells for the TIGR4*cps* or D39-DΔ strains, which was not present with either the TIGR4 or D39 strains (Fig 4.1 and 4.2). Interestingly, at this time point there were only small differences in the association of TIGR4 and TIGR4*cps* strains with RAW 264.7 cells when opsonised in heat-treated serum or PBS, indicating that the increased association of TIGR4*cps* in serum at this time point requires complement. However, at 60 minutes there was a significant increase in association between RAW cells and TIGR4*cps* opsonised in heat-treated serum and PBS compared to TIGR4 (P<0.001, ANOVA). This indicates that there is also a slower, opsonin-independent effect of the TIGR4 capsule on association with macrophages. Overall, similar results for phagocytosis by RAW 264.7 cells were obtained with the D39 strains, with a large early increase in association for D39-DΔ. There was a more significant effect of heat treated serum on association of D39-DΔ with RAW cells (P<0.001, ANOVA) at 15 minutes than seen with the TIGR4 strains (Fig 4.2). In general, both the TIGR4 and D39 strains were resistant to phagocytosis by RAW 264.7 cells, with a maximum of only 10% of cells becoming associated with *S. pneumoniae* within 60 minutes (Fig 4.1 and 4.2).

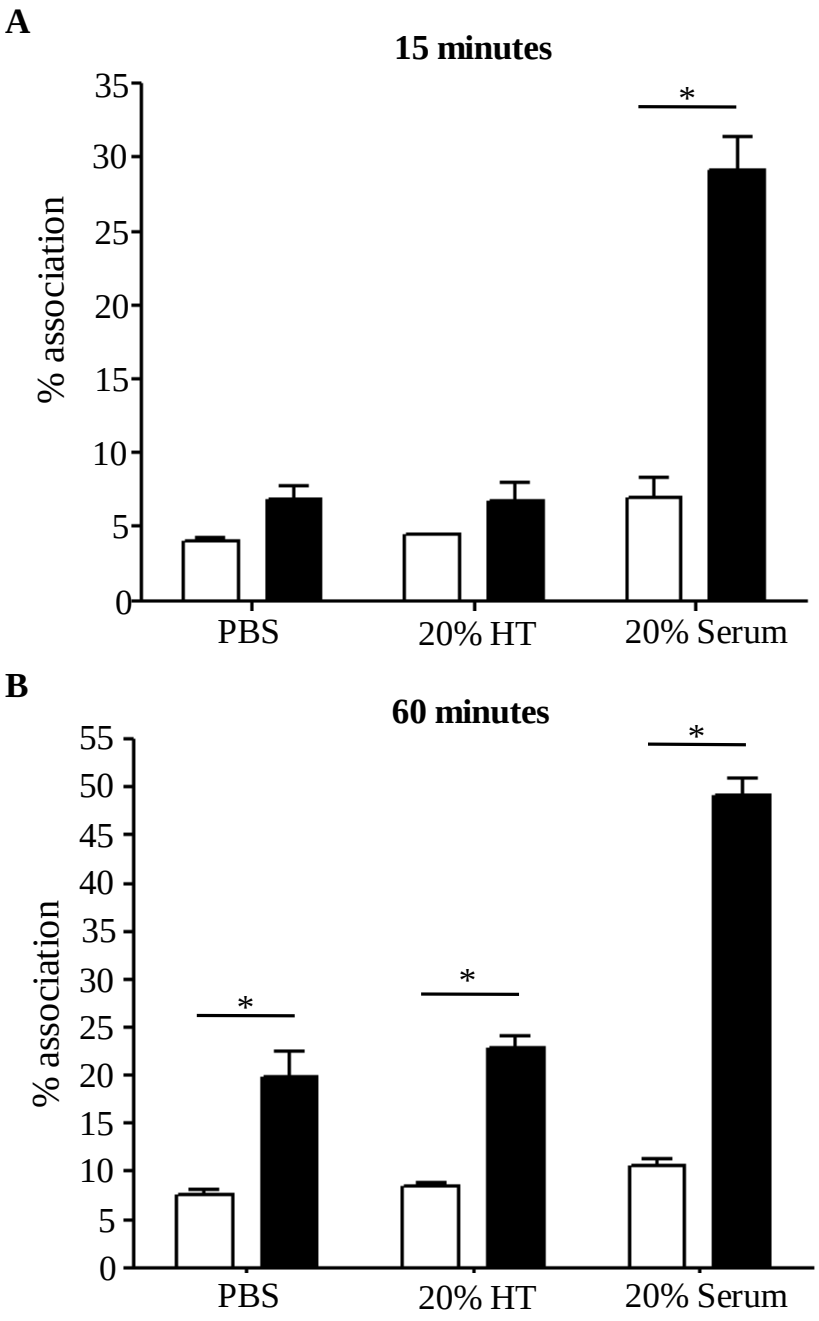
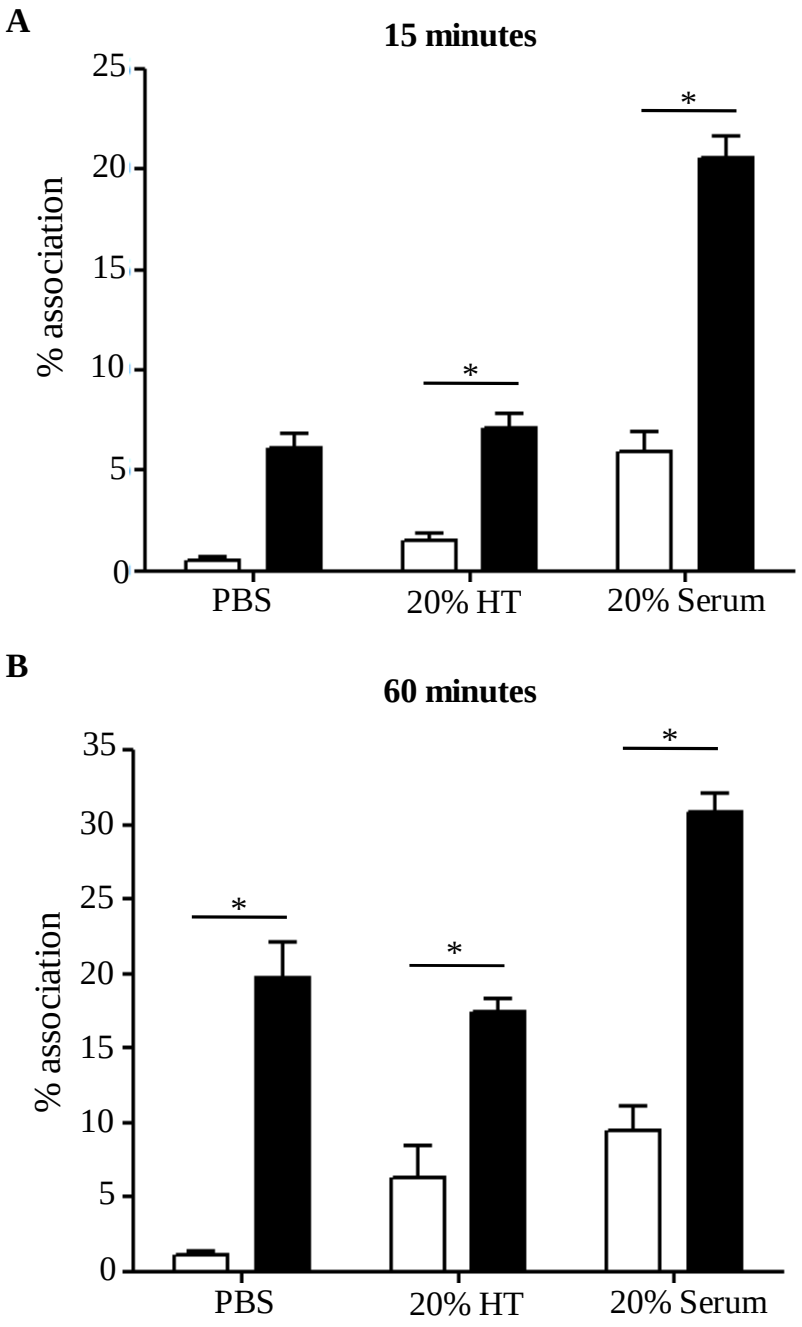


Fig 4.1 RAW 264.7 macrophage phagocytosis of TIGR4 *S. pneumoniae* strains
(A), (B) Percentage of RAW 264.7 cells associated with TIGR4 (open bars) or TIGR4*cps* (solid bars) *S. pneumoniae* at 15 minutes (A) or 60 minutes (B) after opsonisation with HBSS, 20% heat treated (HT) or 20% human serum. For both panels, error bars represent SDs, and * $P < 0.001$ (ANOVA with post-hoc tests).



<u>Fig 4.2</u> RAW 264.7 macrophage phagocytosis of D39 *S. pneumoniae* strains
(A), (B) Percentage of RAW 264.7 cells associated with D39 (open bars) or D39-DΔ (solid bars) *S. pneumoniae* at 15 minutes (A) or 60 minutes (B) after opsonisation in HBSS, 20% heat treated (HT) or 20% human serum For both panels, error bars represent SDs, and * $P <$ 0.001 (ANOVA with post-hoc tests).

4.2.2 Capsule prevents clearance of *S. pneumoniae* from the lungs in early infection

To examine the effect of capsule in early lung infection, 5 x 10^5 CFU FAM-SE labelled *S. pneumoniae* were inoculated IN into CD1 mice in a pneumonia infection model. After 4 hours, bronchoalveolar lavages (BALF) were performed and bacterial CFU in the BALF determined by precise serial dilutions. Even at this early time point there was over a two log reduction in bacterial CFU counts of TIGR4*cps* (Fig 4.3 A) and a one log reduction in D39-DΔ (Fig 4.3 B) compared to their parental strains, indicating a significant survival benefit of the capsule in early lung infection. Furthermore flow cytometry analysis of AMs indicated a significant increase in association of both unencapsulated strains with AMs compared to encapsulated strains observed (TIGR4 strains $P = 0.016$, D39 strains $P = 0.008$ Mann Whitney U-test) (Fig 4.4 A and B). In order to determine if this increase in association between AMs and TIGR4*cps* and D39-DΔ was due to increased cell surface adherence or also represented increased internalisation, the AMs from mice inoculated with the TIGR4 strains were stained with F4/80 and examined with confocal microscopy. Only a small number of AMs were preserved through to visualisation with the confocal microscope, however there were not only more TIGR4*cps* bacteria associated with each AM (TIGR4*cps* 6 IQR 3 versus TIGR4 3 IQR 2), but also an greater proportion of the associated TIGR4*cps* bacteria were internalised in comparison with TIGR4 *S. pneumoniae* (TIGR4*cps* 4 IQR 2 versus TIGR4 1 IQR 2) (Fig 4.5).

TNFα is an important pro-inflammatory cytokine released early in the immune response to bacterial infection, and is important in neutrophil recruitment to the lungs. Hence, the effect of the capsule on the inflammatory response was investigated by measuring TNFα levels in the BALF. Despite the decreased survival of unencapsulated strains, there was more TNFα

in the BALF from mice infected with TIGR4*cps* or D39-DΔ compared to mice infected with the encapsulated, parental TIGR4 or D39 (TIGR4 P = 0.032, D39 strains P = 0.019 Mann Whitney U-test) (Fig 4.6 A and B).

The role of complement on the interactions with the *S. pneumoniae* capsule and AMs in early lung infection was investigated using $C3^{+/-}$ and wild-type C57BL/6 mice. Both strains of mice were intranasally inoculated with 5 x 10^5 CFU FAM-SE labelled TIGR4*cps* or TIGR4 and harvested at 4 hours. Only a maximum of 2000 CFU TIGR4*cps* were recoverable from BALF of WT mice, whereas 2.75 $\log_{10}$ CFU TIGR4*cps* were recoverable from complement deficient mice (Fig 4.7 A). However, there was also an increase in the number of TIGR4 bacteria recovered from the lavage fluid of $C3^{+/-}$ mice over that recovered from WT mice, although this increase was not as marked as that seen with the TIGR4*cps* strain (Fig 4.7 A). There was also an increase in the association between the TIGR4*cps* strain and AMs from $C3^{+/-}$ mice over AMs from wild-type mice (P=0.004, Mann-Whitney U-Test), which was not accompanied by a similar increase in the TIGR4 strain (Fig 4.7 B). In parallel with previous results, TNFα levels were increased in the BALF from mice inoculated with TIGR4*cps* than in mice inoculated with TIGR4. There was also a lower level of TNFα production in $C3^{+/-}$ mice inoculated with TIGR*cps* than found in BALF from wild-type mice (P=0.030, Mann-Whitney U-Test). However, there was no significant difference in TNFα level between WT and $C3^{+/-}$ mice inoculated with TIGR4 (Fig 4.8).

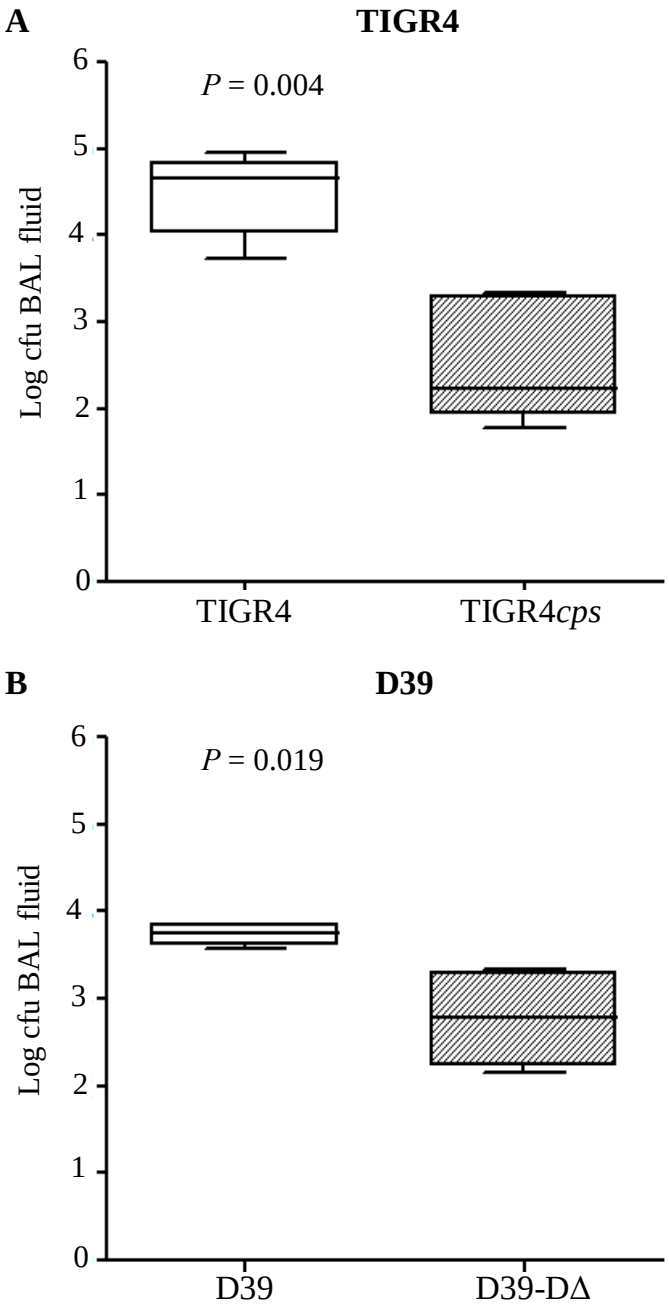


Fig 4.3 Capsule increases survival of *S. pneumoniae* in early lung infection
(A), (B) Bacterial survival counts from BALF at 4 hours after intranasal inoculation of 5 x 10^5 CFU TIGR4 (A) or D39 (B) *S. pneumoniae* strains into CD1 mice. For both panels, open boxes represent encapsulated strains and slashed boxes represent results for unencapsulated strains. *P* values are obtained using Mann-Whitney U-tests.

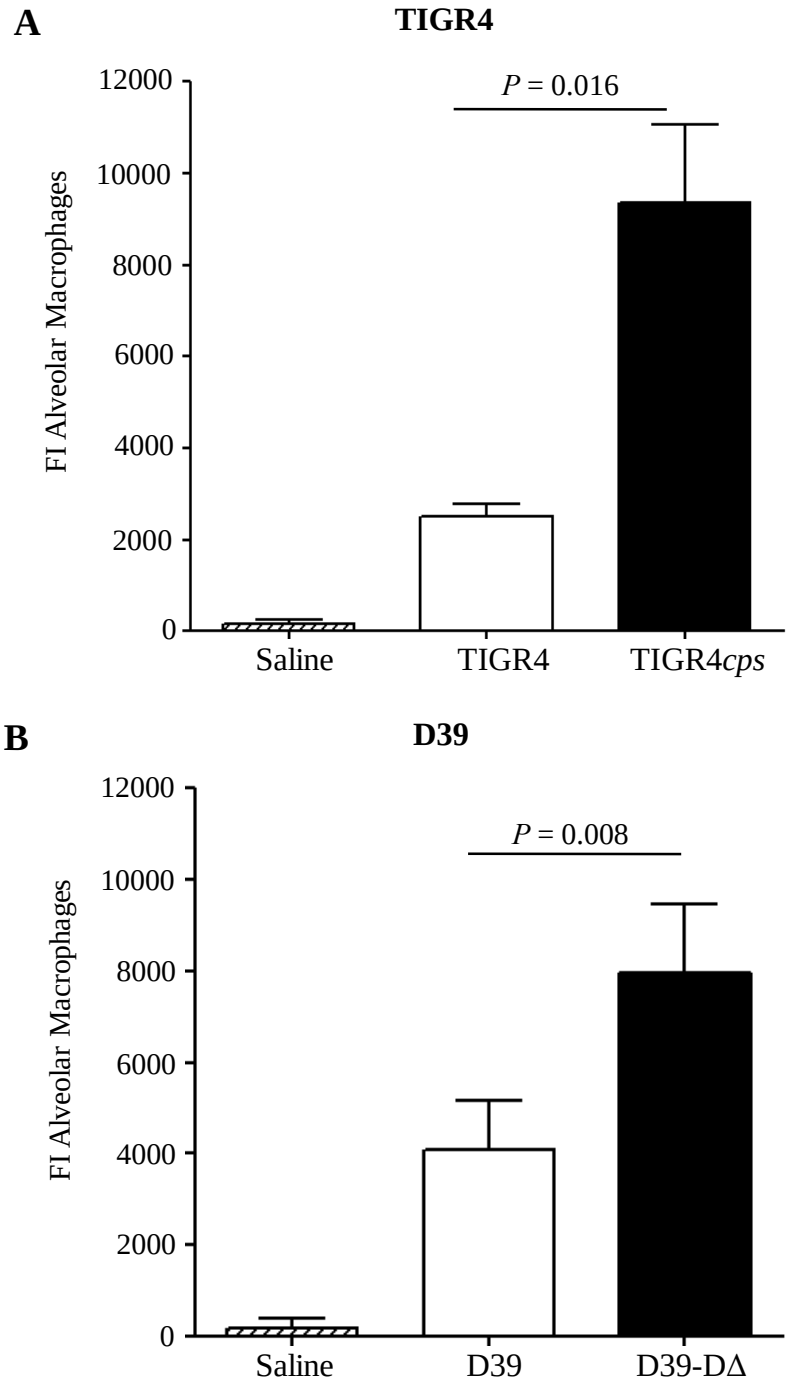
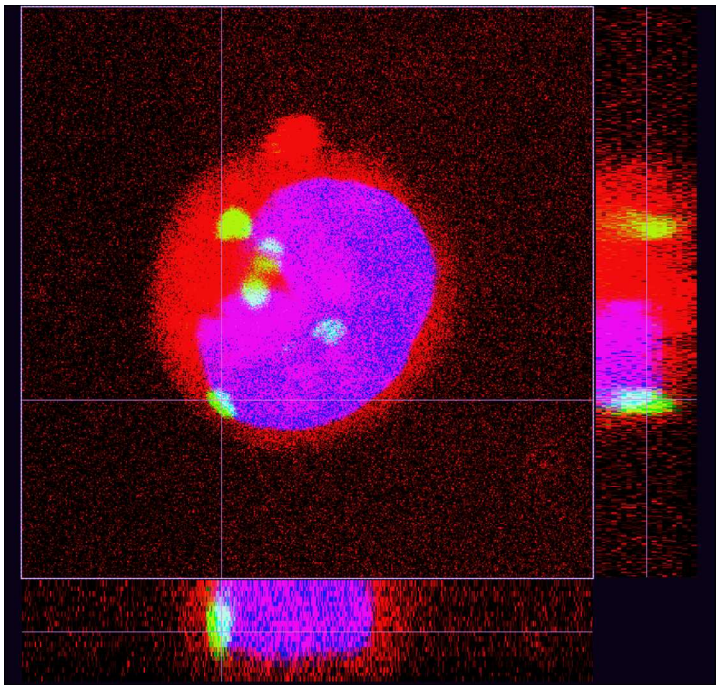
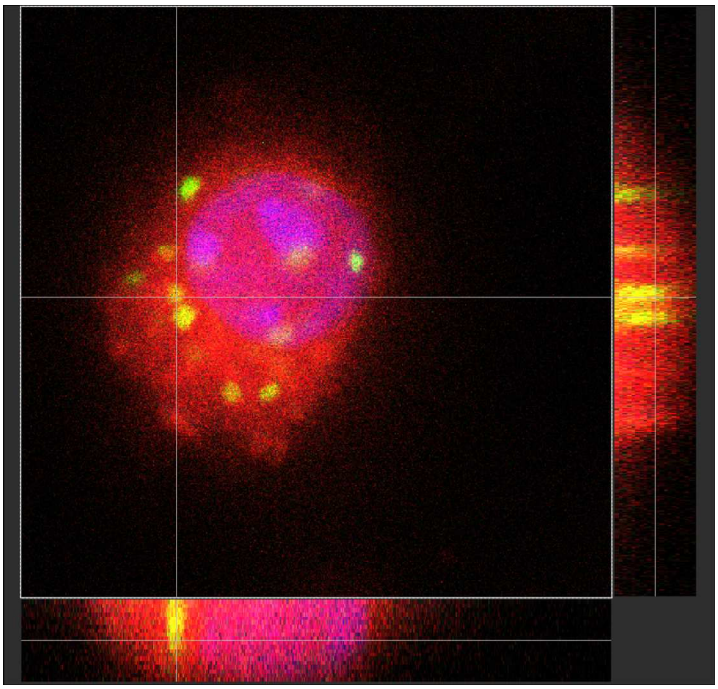


Fig 4.4 Association of *S. pneumoniae* with AMs in early lung infection
(A), (B) Fluorescence of AMs obtained from BALF following 4 hours intranasal infection with 5 x 10^5 CFU FAM-SE labelled TIGR4 (A) and D39 (B) strains, as determined by flow cytometry. For both panels, diagonally slashed bars represent fluorescent index of AMs from mice inoculated with PBS, open bars indicate results from encapsulated strains and closed bars represent results from unencapsulated strains. Error bars represent SDs, and *P* values were obtained using Mann-Whitney U-tests.

A



B



<u>Fig 4.5</u> Confocal microscopy of AMs from infected mice
(A), (B) Representative confocal microscope Z-stack images of AMs obtained from BALF of CD1 mice infected with TIGR4 (A) or TIGR4*cps* (B). For both panels, blue staining represents DAPI, red represents F4/80 and green indicates the FAM-SE labelled *S. pneumoniae*.

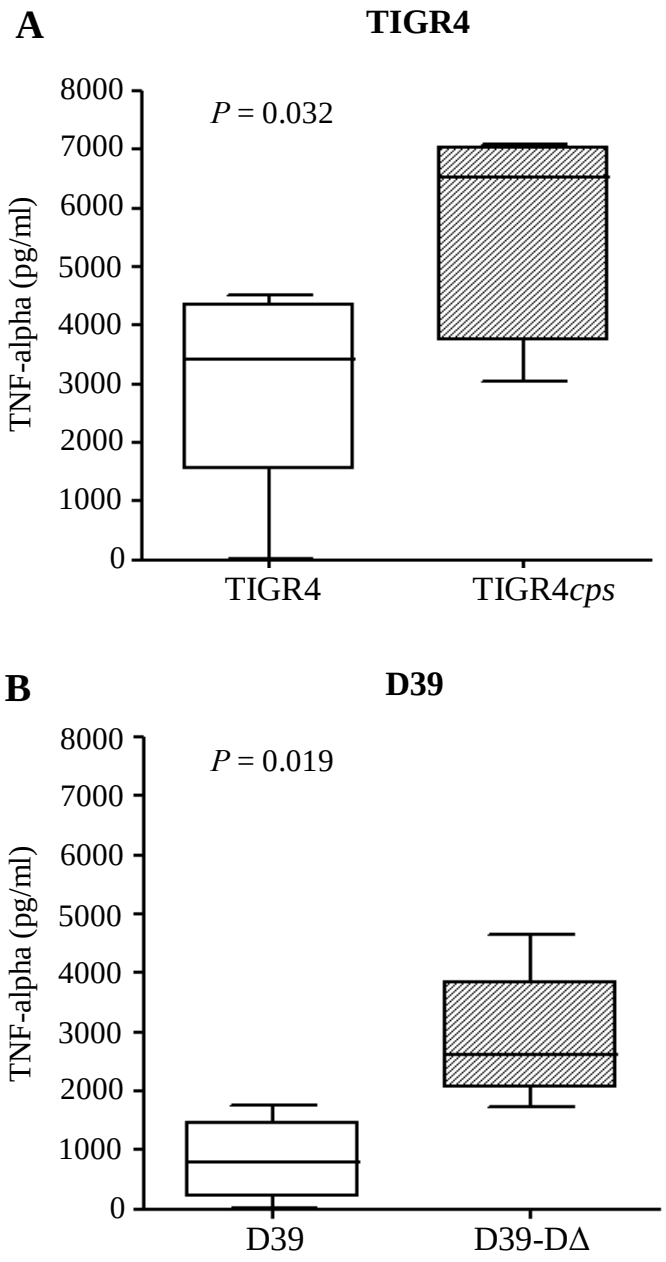


Fig 4.6 The capsule reduces TNFα release during early lung infection
(A), (B) TNFα levels in BALF following 4 hours intranasal infection with TIGR4 (A) and D39 (B) *S. pneumoniae* strains, as determined by ELISA. For both panels, open boxes represents encapsulated strains and slashed boxes unencapsulated strains, with *P* values are obtained using Mann-Whitney U-tests.

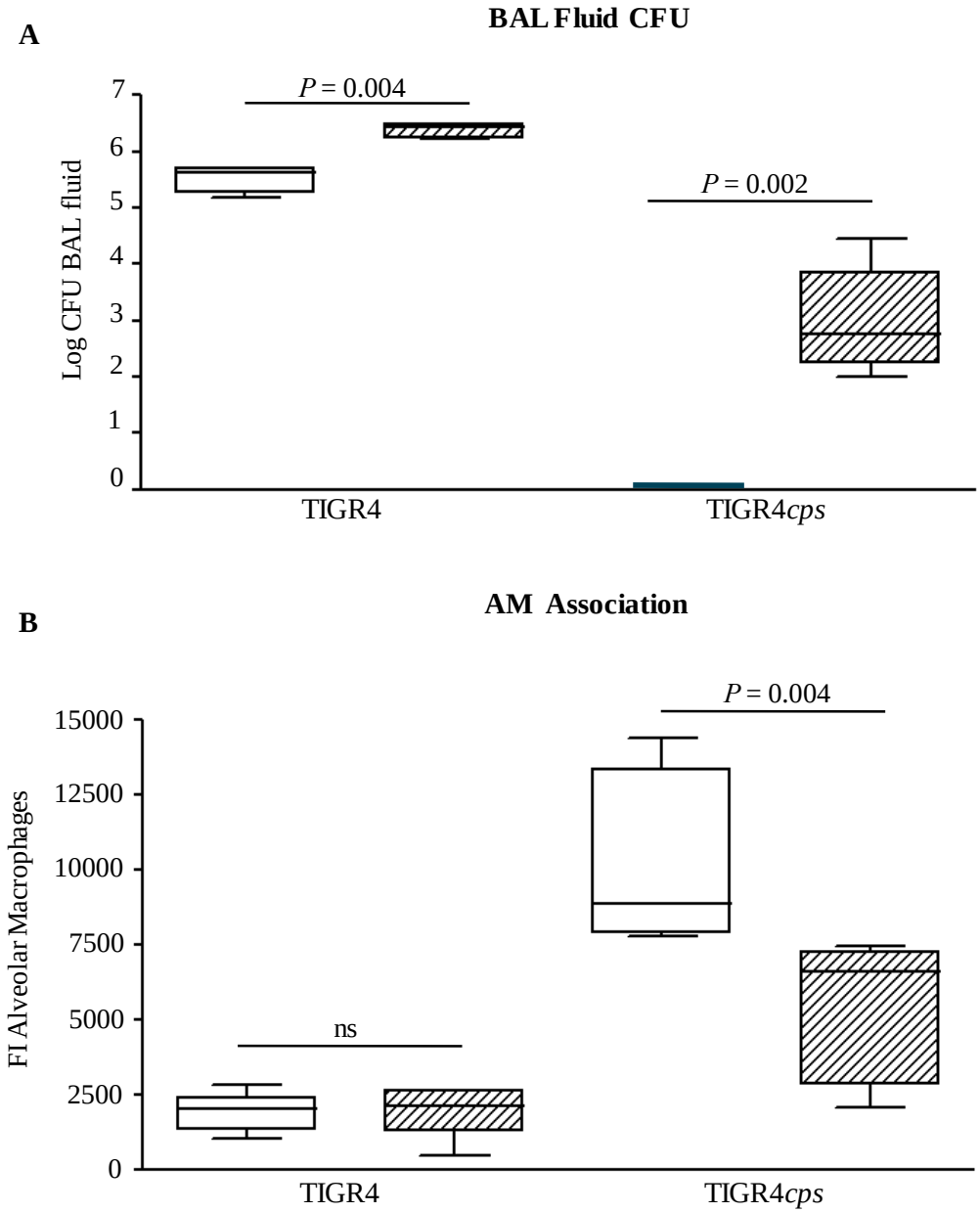
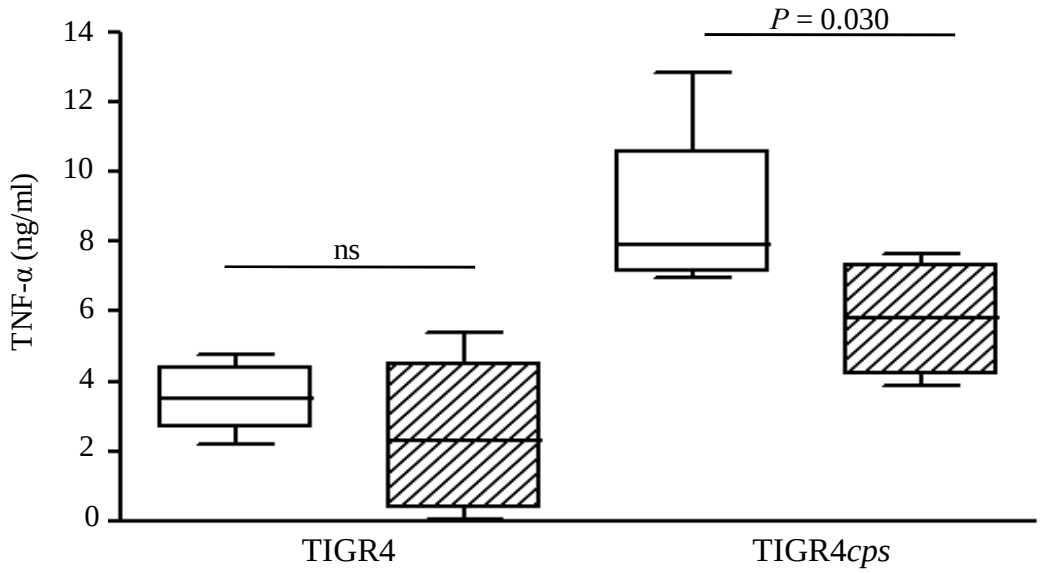


Fig 4.7 The effect of the TIGR4 capsule and complement in early lung infection
(A), (B) Bacterial CFU counts (A) and FI of AMs (B) as determined by flow cytometry on BAL fluid 4 hours after IN infection with FAM-SE labelled TIGR4 or TIGR4*cps* strains into C57BL/6 wild-type (open bars) or $C3^{+/-}$ (diagonal slashed bars) mice. *P* values are derived from Mann-Whitney U-tests.



<u>Fig 4.8</u> The effect of the TIGR4 capsule and complement on TNFα release in early lung infection

TNFα levels in BALF following 4 hours intranasal infection with FAM-SE labeled TIGR4 or *TIGR4cps* strains into C57BL/6 wild-type (open bars) or $C3^{+/-}$ mice (diagonal slashed bars), as determined by ELISA. *P* values are derived from Mann-Whitney U-tests.

4.2.3 The capsule modulates TNFα production by RAW 264.7 cells

The observed inhibition of TNFα release induced by the presence of the capsule in the early lung infection model was investigated further using RAW 264.7 cells *in vitro*. *S. pneumoniae* strains which were pre-opsonised in either PBS, 20% heat treated or 20% normal human serum were incubated with RAW 264.7 cells at a MOI of 10. At 3 hours there was a serum independent release of TNFα from RAW 264.7 macrophages stimulated with the TIGR4*cps* strain, which was not present in macrophages stimulated with TIGR4 (Fig 4.9 A). TNFα production at 24 hours indicated that there was a greater response to TIGR4*cps* in all opsonising conditions, and that there was a large complement dependent effect, as shown by the difference between TIGR4*cps* opsonised with heat-treated and normal human serum. Furthermore, there was very little TNFα production stimulated by the TIGR4 strain over 24 hours with any opsonin, indicating the ability of the TIGR4 capsule to inhibit TNFα production by macrophages (Fig 4.9 B). The D39 strains showed a similar response, although with a smaller complement dependent effect observable at 24 hours in macrophages stimulated with D39-DΔ.

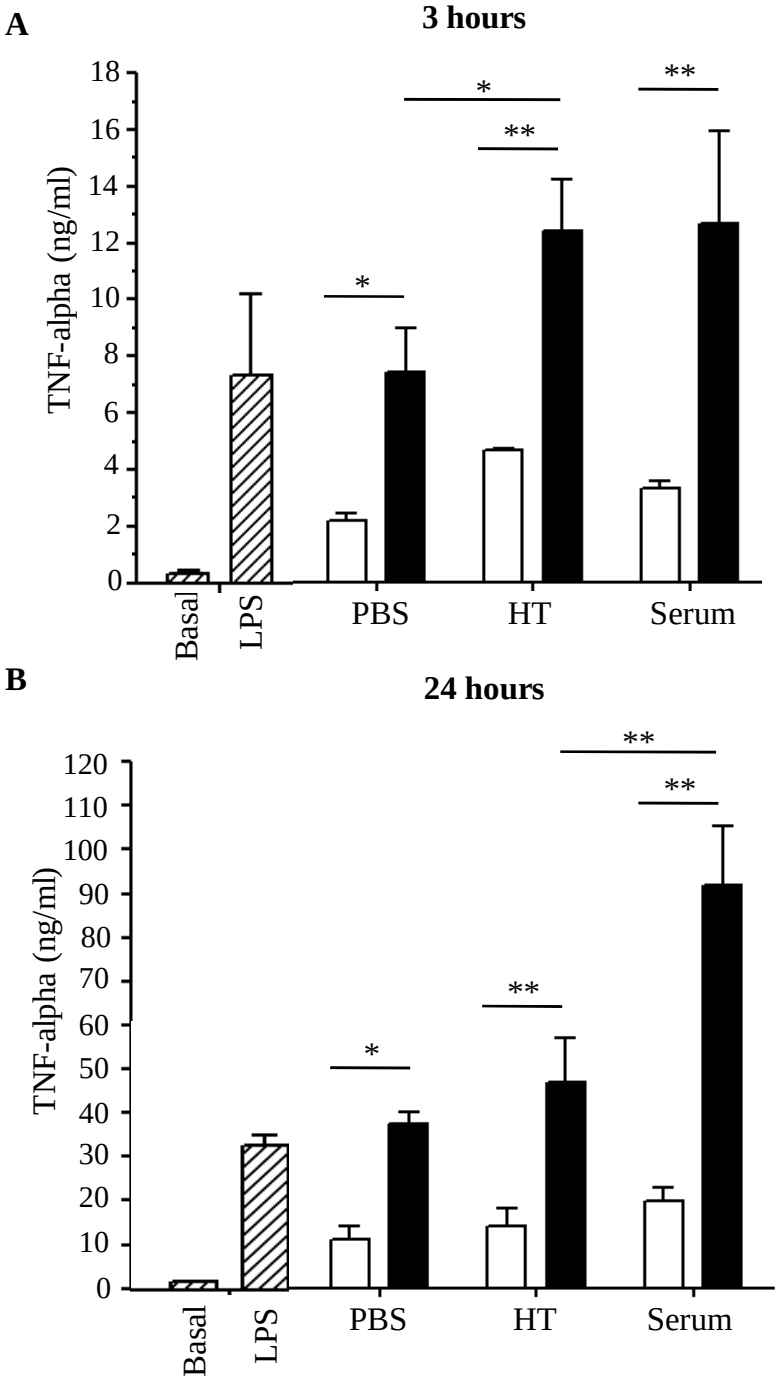


Fig 4.9 TNF*α* production by RAW 264.7 cells stimulated with TIGR4 strains
(A), (B) TNFα release from 1 x 10^6 RAW 264.7 cells stimulated with 100 ng/ml LPS or MOI 10 TIGR4 or TIGR4*cps* opsonised with PBS, heat treated 20% serum (HT) or 20% serum for 3 hours (A) or 24 hours (B) as measured by ELISA. For both panels, error bars represent SDs and * P<0.01 or ** P<0.001 (ANOVA with post-hoc tests).

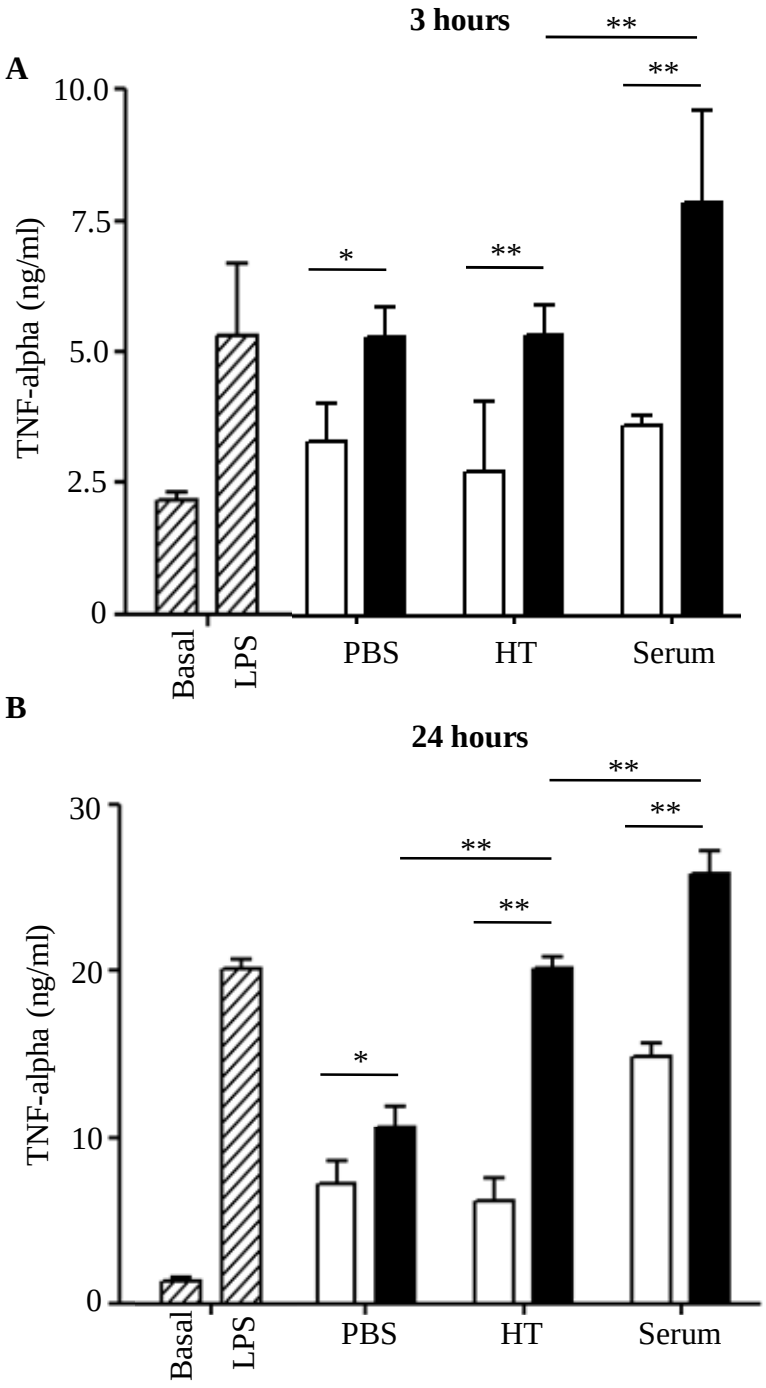
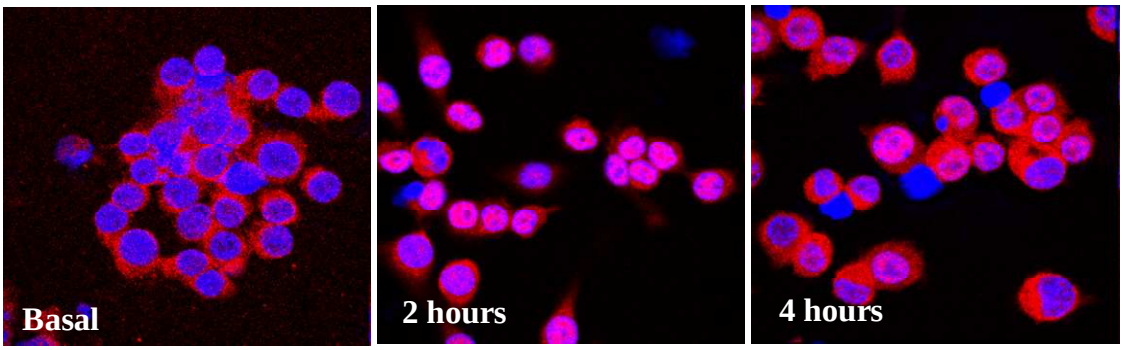


Fig 4.10 TNFα production by RAW 264.7 cells stimulated with D39 strains
(A), (B) TNFα release from 1 x 10^6 RAW 264.7 cells stimulated with 100 ng/ml LPS or MOI 10 D39 or D39-DΔ opsonised with PBS, heat treated 20% serum (HT) or 20% serum after 3 hours (A) or 24 hours (B) as measured by ELISA. For both panels, error bars represent SDs and * P<0.01 or ** P<0.001 (ANOVA with post-hoc tests).

4.2.4 The *S. pneumoniae* capsule inhibits NFκB pathways activation of macrophages

TNFα production is stimulated through the NFκB activation pathway. Hence quantitative confocal immunofluorescence assays of NFκB RelA (p65) nuclear translocation were performed to investigate innate immune cellular activation by the encapsulated and unencapsulated strains. To follow the time course of NFκB translocation in activated RAW 264.7 cells they were stimulated with 100ng/ml LPS over 4 hours. RelA staining was mostly cytoplasmic in control cells, and in response to LPS reached the greatest nuclear staining at 2 hours (Fig 4.11). In order to determine if *S. pneumoniae* stimulated NFκB translocation, the study was repeated selecting a 1 hour time point. Both TIGR4 and TIGR4*cps* show greater nuclear NKκB staining than unstimulated RAW 264.7 cells, with an apparent increase in NKκB translocation present in cells stimulated with TIGR4*cps* (Fig 4.12). This was investigated further by stimulating RAW 264.7 cells with increasing MOIs of 20% serum opsonised *S. pneumoniae* in a dose response study. Greater nuclear staining was evident in response to increasing MOIs of *S. pneumoniae*, and quantitative image analysis found that there was increased NFκB translocation in RAW 264.7 cells stimulated with TIGR4*cps* and D39-DΔ than TIGR4 or D39 (Fig 4.13 A and B). To see if this increase in innate activation was complement dependent or independent, RAW 264.7 cells were incubated with MOI 10 *S. pneumoniae* which had been opsonised in HBSS, 20% heat-treated or 20% normal human serum. In all opsonins, unencapsulated *S. pneumoniae* induced increased NFκB nuclear translocation for both the TIGR4 and D39 strains (Fig 4.14 A and B).

A



B

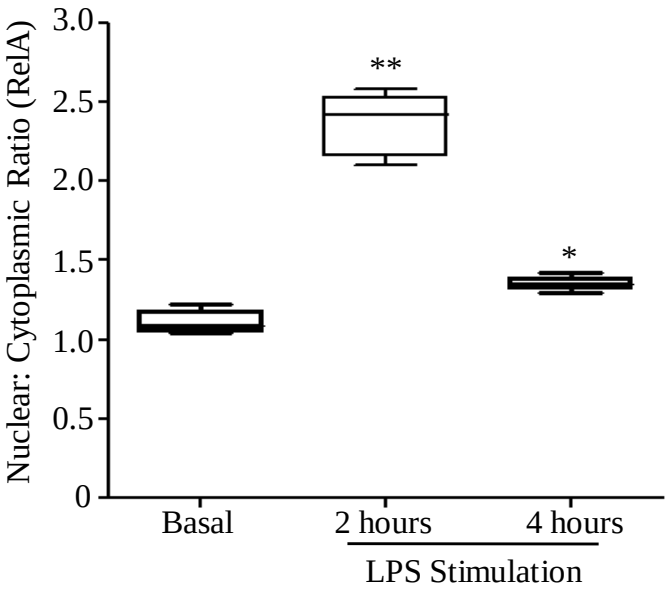


Fig 4.11 Time course study of NFκB translocation in RAW 264.7 cells
(A) Sample confocal microscopy images of RAW 264.7 cells when unstimulated or activated with 100ng/ml LPS for either 2 or 4 hours and stained for RelA (p65) (red). DAPI was used to stain the nucleus. (B) Quantification of nuclear:cytoplasmic RelA staining in a time course study of NFκB translocation in LPS stimulated RAW 264.7 cells. * $P < 0.01$ or ** $P < 0.01$ for stimulated cells compared to unstimulated cells (Kruskal-Wallis with Dunn's multiple comparison test).

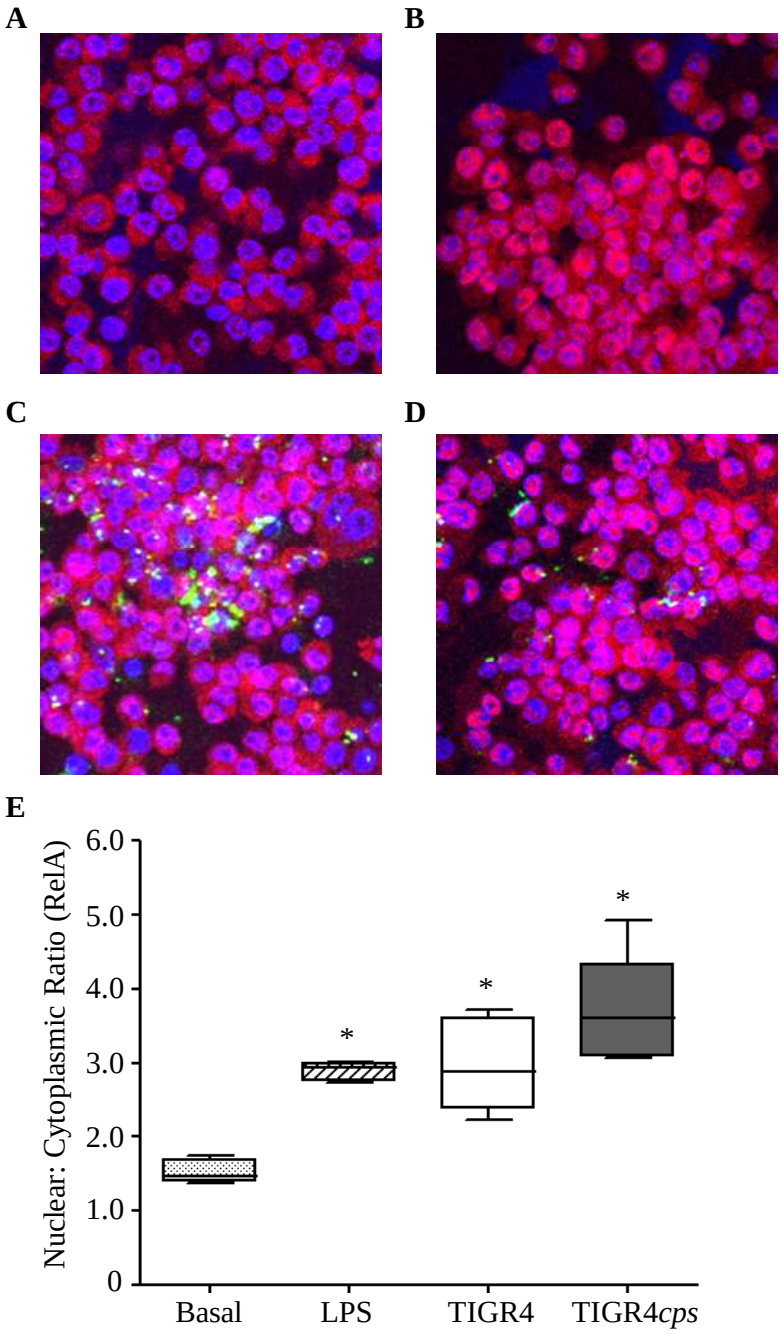
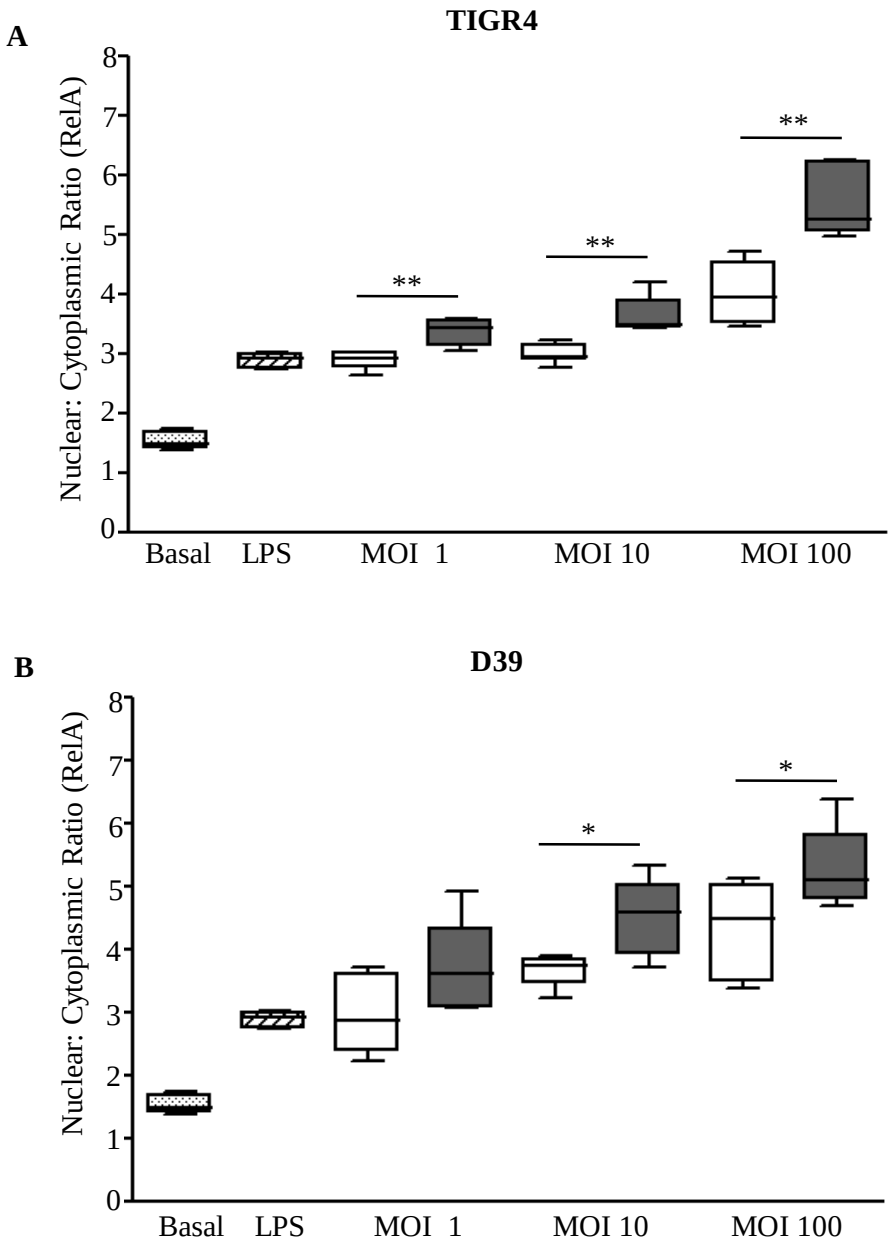


Fig 4.12 NFκB translocation in RAW 264.7 cells stimulated with TIGR4 and TIGR4*cps*

(A) to (D) Sample confocal microscopy images of RAW 264.7 cells when unstimulated (A) or activated with 100ng/ml LPS (B), MOI 10 TIGR4 (C) or TIGR4*cps* (D) for 1 hour. (E) Quantification of nuclear:cytoplasmic RelA (p65) staining in a 1 hour study of NFκB translocation in LPS or *S. pneumoniae* stimulated RAW 264.7 cells. * $P < 0.01$ for stimulated cells compared to unstimulated cells (Kruskal-Wallis with Dunn's multiple comparison test).



<u>Fig 4.13</u> Dose response of NFκB translocation in RAW 264.7 cells stimulated with *S. pneumoniae*

(A), (B) Quantification of nuclear:cytoplasmic RelA (p65) staining in a dose-response study of NFκB translocation in RAW 264.7 cells stimulated with 100ng/ml LPS (slashed bars) or *S. pneumoniae* TIGR4 (A) or D39 (B) encapsulated strains (open bars) or unencapsulated strains (closed bars). For both panels, * $P < 0.05$ or ** $P < 0.01$ (Mann-Whitney U-test), and all stimulations produced significant NFκB translocation compared to unstimulated (basal) cells (Kruskal-Wallis with Dunn's multiple comparison test).

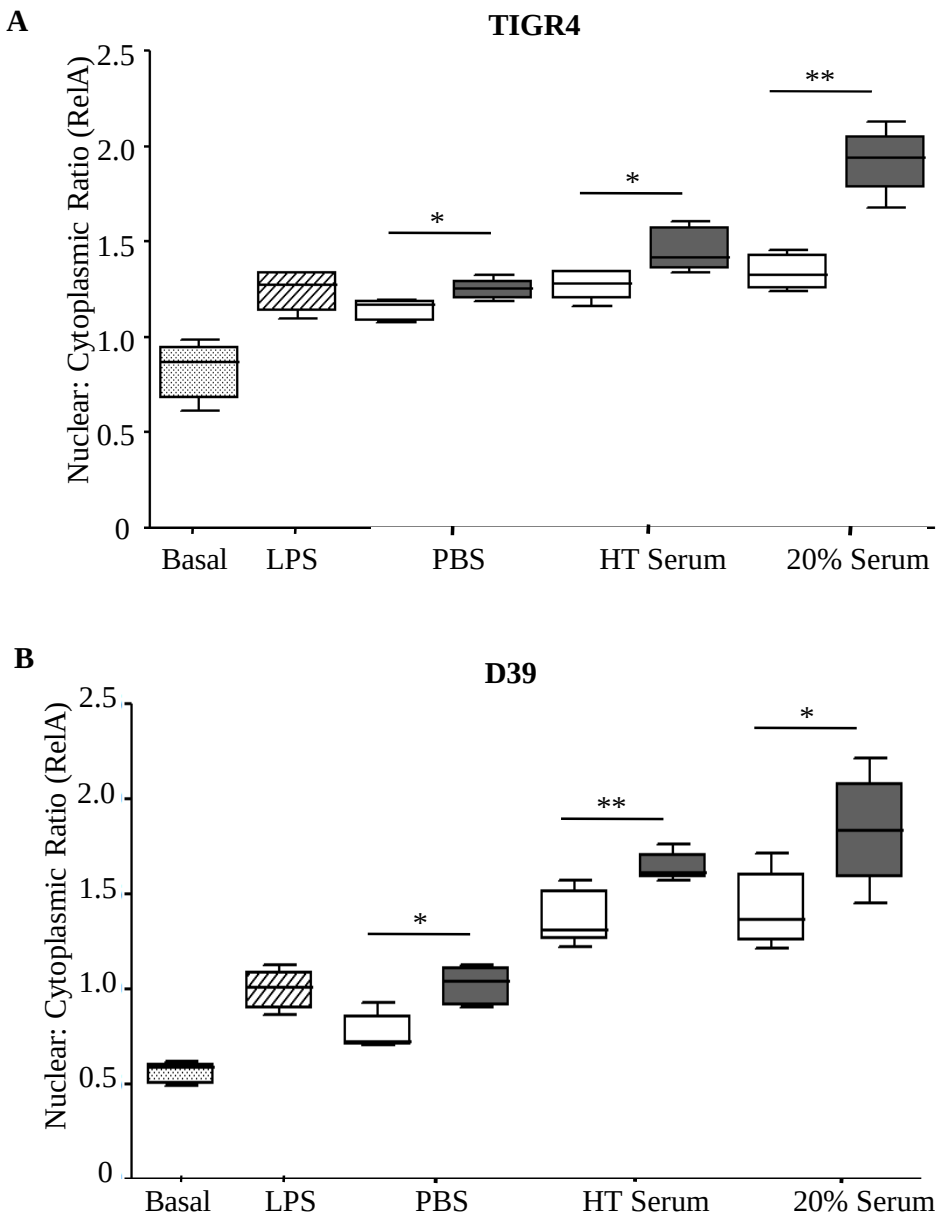


Fig 4.14 Complement dependent and independent NFκB translocation in RAW 264.7 cells

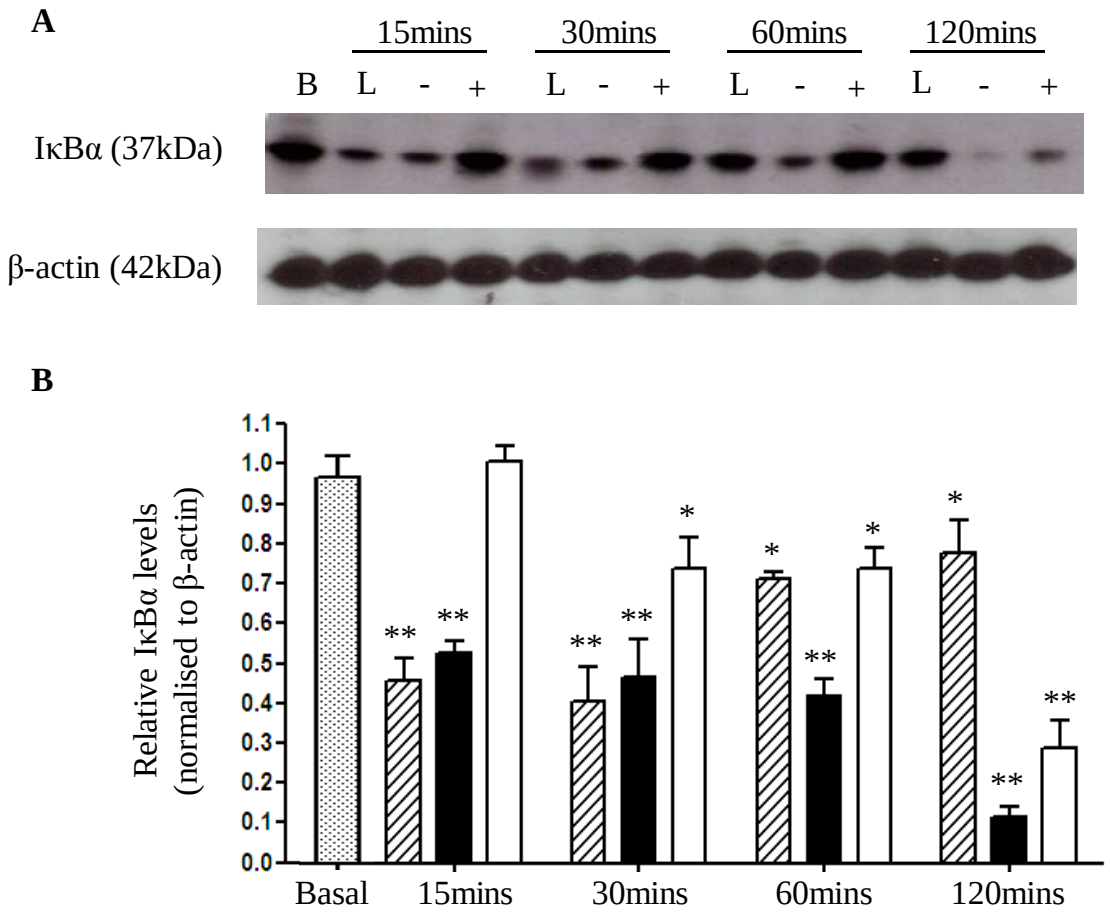
(A), (B) Quantification of nuclear:cytoplasmic RelA (p65) staining in a study of NFκB translocation in RAW 264.7 cells stimulated with 1ng/ml LPS (slashed bars) or *S. pneumoniae* TIGR4 (A) or D39 (B) encapsulated strains (open bars) or to unencapsulated strains (closed bars) opsonised in PBS, heat-treated (HT) 20% serum or 20% serum. For both panels, * $P < 0.05$ or ** $P < 0.01$ (Mann-Whitney U-test), and all stimulations produced significant NFκB translocation compared to unstimulated (basal) cells (Kruskal-Wallis with Dunn's multiple comparison test).

4.2.5 Innate immune cellular activation of RAW 264.7 cells by TIGR4 and TIGR4*cps*

Classically, IκBα is phosphorylated by IκB kinase and is degraded by ubiquitination, allowing for nuclear translocation of NFκB complex. Degradation of IκBα can be detected by western blotting; hence IκBα degradation was examined in a time course study of RAW 264.7 cells stimulated with TIGR4 and TIGR4*cps* which were previously opsonised in 20% human serum. 100ng/ml LPS was used as a control to monitor the activation of these pathways in RAW 264.7 cells. In RAW 264.7 cells that were stimulated with LPS the IκBα signal was diminished at 15 and 30 minutes, with the signal increasing in strength over the next 90 minutes. RAW 264.7 cells stimulated with TIGR4*cps* also showed rapid degradation of IκBα within 15 minutes, and the signal intensity remained the same at 30 and 60 minutes, and then decreased by 120 minutes. However, RAW 264.7 cells stimulated with TIGR4 did not show a reduced IκBα signal at 15 minutes, with IκBα signal only reducing at 30 minutes. At all time points studied, there was a stronger IκBα signal in RAW 264.7 cells stimulated with TIGR4 than with TIGR4*cps* ($P<0.001$ ANOVA with post hoc tests) (Fig 4.15), indicating an attenuation of IκBα degradation in TIGR4 stimulated macrophages, supporting the results of the NFκB translocation assay (Fig 4.13).

Since pneumococcal components can activate Toll like receptors (TLRs) it is also likely that macrophage responses to *S. pneumoniae* are affected by mitogen activated protein kinases (MAPKs). These innate immune cellular pathways operate in a network of molecules which converge to a limited number of intracellular signalling events, including the ERK1/2 and p38 signalling pathways which have been shown to be involved in macrophage and epithelial cell inflammatory responses to *S. pneumoniae* (N'Guessan et al. 2006; Kang et al. 2009). Immunoblots were therefore used to study phosphorylation of p38

and ERK 1/2 in RAW 264.7 cells. For both MAP kinases, there was an increase in the level of phosphorylated protein detectable over time on stimulation with *S. pneumoniae*, with phosphorylated ERK 1/2 levels increasing until 60 minutes and then decreasing at 120 minutes (Fig 4.16). Phosphorylated p38 showed a similar pattern, however levels of detectable phosphorylated p38 did not decrease after 60 minutes (Fig 4.17). However, in contrast to the results obtained for IκBα, there was no detectable difference in the level of phosphorylation of either ERK 1/2 or p38 induced by TIGR4 and TIGR4*cps* ($P>0.05$ ANOVA with post-hoc tests) (Fig 4.16 and 4.17). This indicates an NFκB pathway specific effect of the capsule on inhibition of innate immune activation, which is not merely attributable to increased adherence of TIGR4*cps*.



<u>Fig 4.15</u> IκBα degradation in RAW 264.7 cells stimulated with TIGR4 and TIGR4*cps*
(A) Representative western immunoblot of the time course of IκBα degradation at 15, 30, 60 and 120 minutes in response to stimulation with 1ng/ml LPS or MOI 10 TIGR4 (+) or TIGR4*cps* (-). Actin expression is demonstrated in each assay to show equivalent sample loading. (B) Analysis of western immunoblots of IκBα degradation from triplicate experiments. Slashed bars represent samples from LPS stimulated RAW 264.7 cells, open bars TIGR4 and closed bars represent TIGR4*cps*. Error bars represent SDs, * $P<0.01$ or ** $P<0.001$ (ANOVA with post-hoc tests) for differences between basal IκBα. There was a significant difference in IκBα levels in the TIGR4 and TIGR4*cps* stimulated RAW 264.7 cells at all time points ($P < 0.001$, ANOVA with post-hoc tests).

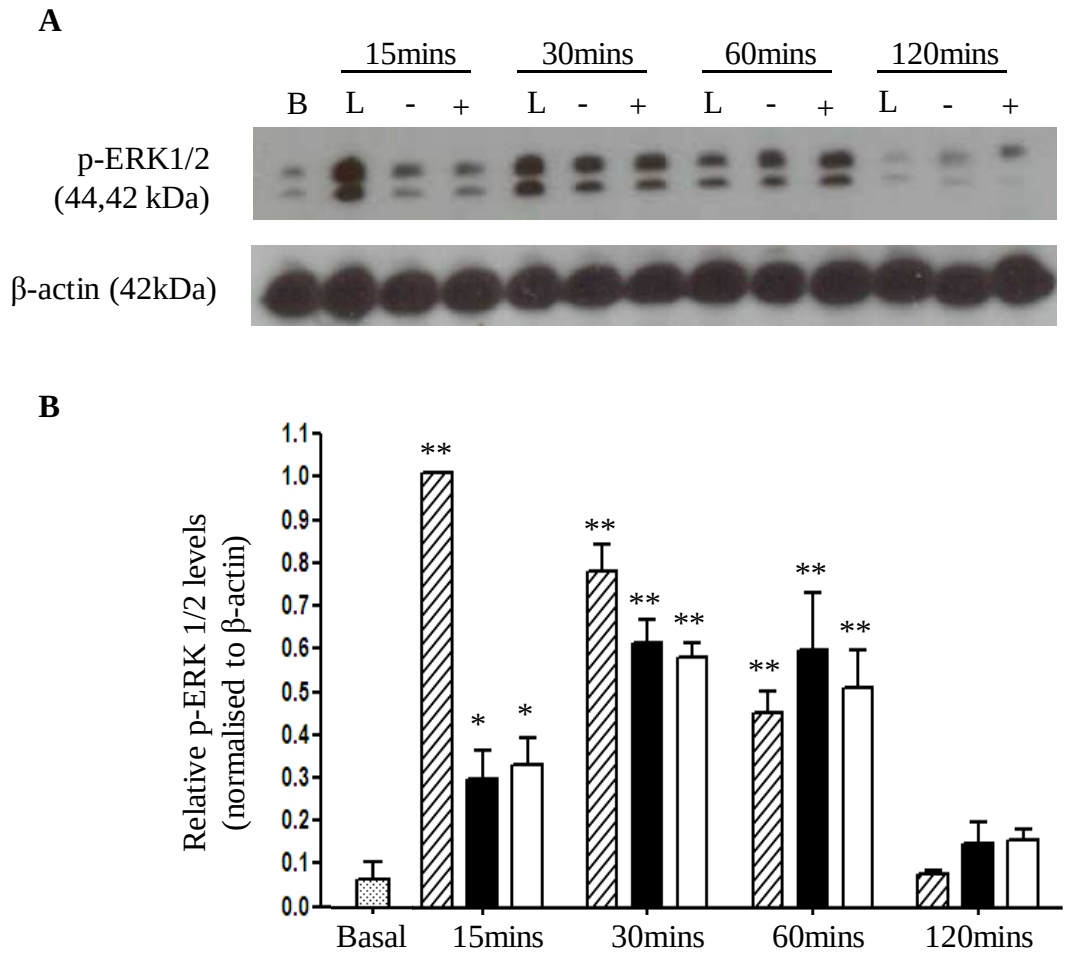


Fig 4.16 Phosphorylation of ERK 1/2 in RAW 264.7 cells stimulated with TIGR4 and TIGR4*cps*

(A) Representative western immunoblot of the time course of phosphorylation of ERK1/2 at 15, 30, 60 and 120 minutes in response to stimulation with 1ng/ml LPS or MOI 10 TIGR4 (+) or TIGR4*cps* (-). Actin expression is demonstrated in each assay to show equivalent sample loading. (B) Analysis of western immunoblots of ERK1/2 phosphorylation from triplicate experiments. Slashed bars represent samples from LPS stimulated RAW 264.7 cells, open bars TIGR4 and closed bars represent TIGR4*cps*. Error bars represent SDs, * $P<0.01$ or ** $P<0.001$ (ANOVA with post-hoc tests) for differences between basal ERK1/2. There was no significant difference in phosphorylated ERK1/2 in the TIGR4 and TIGR4*cps* stimulated RAW 264.7 cells at all time points (ANOVA with post-hoc tests).

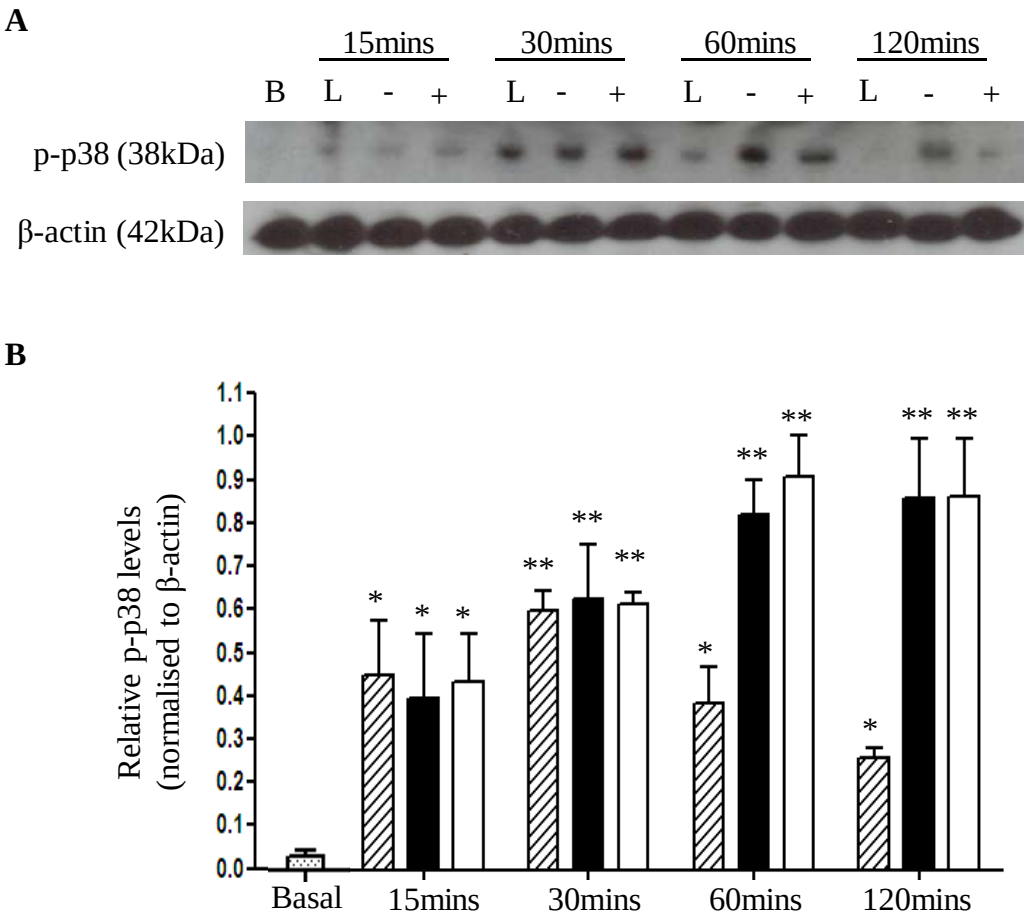


Fig 4.17 Phosphorylation of p38 in RAW 264.7 cells stimulated with TIGR4 and TIGR4*cps*

(A) Representative western immunoblot of the time course of phosphorylation of p38 at 15, 30, 60 and 120 minutes in response to stimulation with 1ng/ml LPS or MOI 10 TIGR4 (+) or TIGR4*cps* (-). Actin expression is demonstrated in each assay to show equivalent sample loading. (B) Analysis of western immunoblots of p38 phosphorylation from triplicate experiments. Slashed bars represent samples from LPS stimulated RAW 264.7 cells, open bars TIGR4 and closed bars represent TIGR4*cps*. Error bars represent SDs, * $P<0.01$ or ** $P<0.001$ (ANOVA with post-hoc tests) for differences between basal p38. There was no significant difference in phosphorylated p38 in the TIGR4 and TIGR4*cps* stimulated RAW 264.7 cells at all time points (ANOVA with post-hoc tests).

4.3 SUMMARY

In summary, the results in this chapter define some of the effects of capsule with macrophages, both *in vitro* and *in vivo*. The *S. pneumoniae* capsule prevents a rapid complement dependent association with RAW 264.7 macrophages, and there is also a slower complement independent capsular effect in both the TIGR4 and D39 strains. This is also apparent in early lung infection, where the capsule decreases association with AMs at an early time point and increases *S. pneumoniae* survival. Interestingly, despite the decreased bacterial survival, there is an increased TNFα inflammatory response within 4 hours of infection in BALF from mice infected with either TIGR4*cps* or D39-DΔ. Bacterial survival and the increased association of AMs in TIGR4*cps* is partially dependent on complement, although these results are difficult to interpret as there is also increased survival and decreased AM association in the TIGR4 strain.

Further investigation of the innate inflammatory response of macrophages using RAW 264.7 cells showed that both TNFα release at 3 hours and production within 24 hours are dependent on complement, with increased TNFα in the supernatant of macrophages stimulated with TIGR4*cps* and D39-DΔ. However there were differences in the level of complement-independent capsule effect between the TIGR4 and D39 strains, and this was matched by the results of quantitative confocal immunofluorescence assays NFκB RelA translocation assays. Furthermore, immunoblot time course studies of IκBα degradation in RAW 264.7 cells stimulated with serum opsonised TIGR4 strains correlated with the confocal NFκB translocation studies. Interestingly, the effect of the capsule on innate immune cellular activation in RAW 264.7 cells seems to be pathway specific, as there is differential activation between TIGR4 and TIGR4*cps* in the NFκB pathway but not the p38

and the ERK 1/2 pathways. However, it remains unclear as to whether this pathway specific effect is dependent on complement and internalisation, and further experiments using different opsonisation conditions and in the presence of cytochalasin are required to define the full effects of capsule on this pathway.

CHAPTER 5

EFFECT OF CAPSULAR SEROTYPE ON COMPLEMENT MEDIATED IMMUNITY

5.1 INTRODUCTION

The mechanisms causing capsular serotype-dependent variation in virulence are largely unknown, but may reflect differences in the ability between serotypes to inhibit host immune responses, and this hypothesis is partially supported by existing experimental data. Different capsular serotypes vary markedly in their virulence in mouse infection models, but since there is a weak relationship between virulence in mice and invasive potential in humans the clinical relevance of these findings is unclear (Briles et al. 1992; Sandgren et al. 2005). Additionally, complement resistance and phagocytosis varies between strains with different capsular serotypes (Winkelstein et al. 1976; Hostetter 1986; Yuste et al. 2008). However there is also considerable genetic variation between *S. pneumoniae* strains outside of the capsule genetic locus, and this genetic variation is partially linked to capsular serotype (http://www.mlst.net/). Therefore, the relationship between capsular serotype and invasiveness could be due to non-capsular genetic variation rather than any direct effects of the polysaccharide capsule. To overcome strain genetic variation confounding the assessment of capsular serotype interactions with the immune system, the capsular loci of one strain can be replaced with the capsular loci from another, creating otherwise isogenic strains expressing different capsular serotypes (Trzcinski et al. 2003; Nelson et al. 2007). Previous studies have established the principle that capsular serotype can affect complement sensitivity and virulence of *S. pneumoniae* independent of strain background (Kelly et al. 1994; Abeyta et al. 2003). However, as yet there are only limited data on the

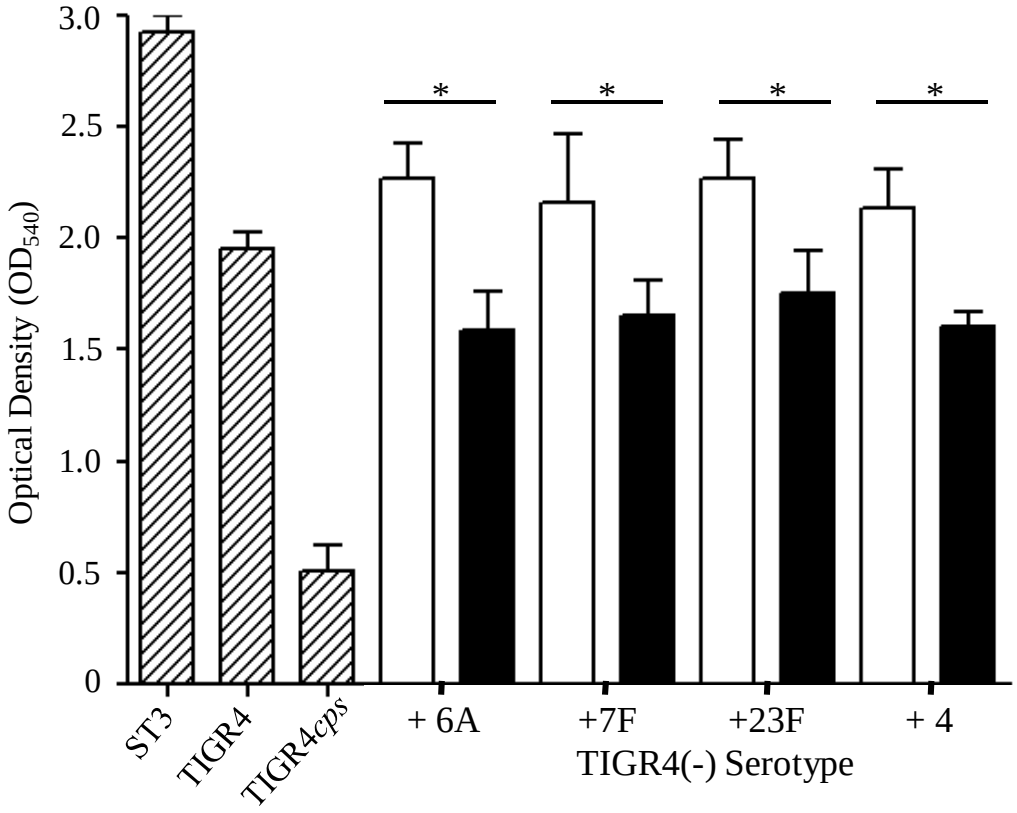
effects of different capsular serotypes on immunity to *S. pneumoniae* and a more detailed assessment is required to help understand why capsular serotype is linked to invasive potential.

In this chapter, I have used TIGR4 *S. pneumoniae* strains modified to express different capsular serotypes, two representative of relatively invasive serotypes (4 and 7F) and two representative of less invasive serotypes (6A and 23F) in order to investigate capsular serotype-dependent effects on immunity. This chapter examines both the effect of capsular serotype and phase variation on complement deposition, neutrophil phagocytosis and virulence in a mouse model of septicaemia using capsular switch TIGR4 strains.

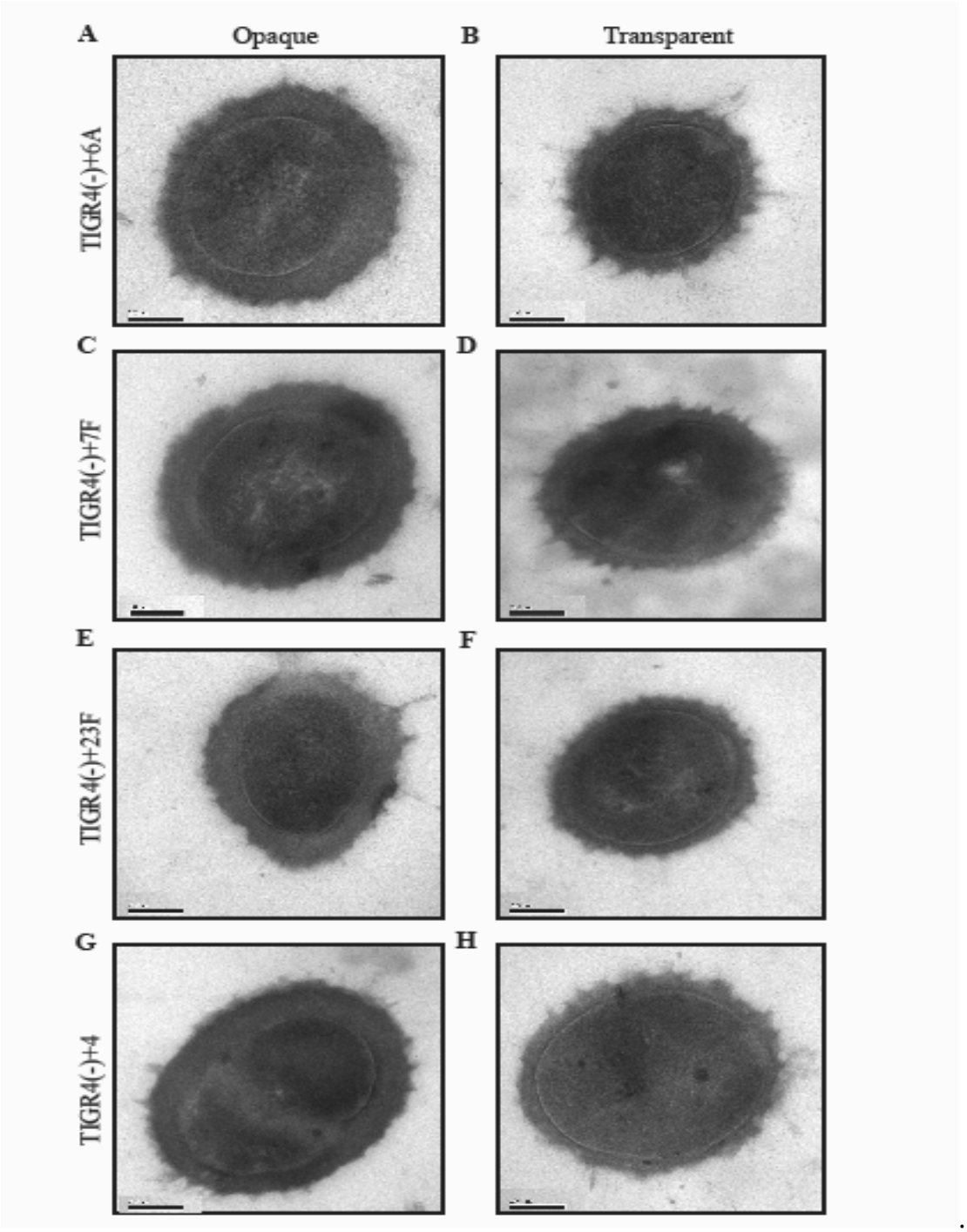
5.2 RESULTS

5.2.1 Measurement of capsule thickness in TIGR4 capsular switch strains

S. pneumoniae TIGR4 strains which were genetically modified at the capsule gene locus but which are otherwise isogenic, were given as a gift from Prof J Weiser, University of Pennsylvania. The TIGR4 capsular switch strains were confirmed to be in either opaque or transparent phase by growth on Tryptone Soy agar with catalase. These strains were then assessed to determine the amount of capsule polysaccharide expressed by each strain. The mucopolysaccharide Stains-All assay revealed all of the opaque strains contained more capsular polysaccharide than the transparent phase variant ($P < 0.001$, Fig 5.1). However, this assay depends on the ratio of anionic sugars to monosaccharides, and is only appropriate for comparisons within a serotype but not between structurally distinct polysaccharides. Therefore, EM was used to measure the average thickness of the capsule polysaccharide. The TIGR4 capsular switch strains were grown to mid-log phase and prepared for electron microscopy using a ruthenium red and lysine acetate protocol to preserve capsule polysaccharide (Hammerschmidt et al. 2005). Image analysis was used to quantitate the area inside the cell wall and the total area of the bacterial cell including the capsule layer, and by making a presumption of circularity the diameter of each was calculated thereby allowing the average capsule thickness to be determined. This method confirmed that the capsules of the opaque strains were indeed thicker than the polysaccharide capsules of the transparent strains (ANOVA $P > 0.001$). Furthermore, there were no significant differences in the thickness of the capsule between the capsular switch strains in either the opaque phase or in the transparent phase (ANOVA $P > 0.05$, Fig 5.2 and Table 5.1).



<u>Fig 5.1</u> Biochemical capsule assessment in TIGR4 capsule switch strains
Results of the Stains-All semi-quantitative assay for the amount of bacterium-associated capsule polysaccharide for strains used in this chapter. Serotype 3 wild-type, and TIGR4 encapsulated and unencapsulated strains (slashed bars) were used as controls and compared to TIGR4(-) capsular switch strains in either opaque (open bars) or transparent (closed bars) phase variants (unpaired student's t-test * $P < 0.001$).



<u>Fig 5.2</u> EM capsule measurement in TIGR4 capsular switch strains
(A)-(H) Representative immunogold EM images of the TIGR4(-) +6A opaque (A) and transparent (B), +7F opaque (C) and transparent (D), +23F opaque (E) and transparent (F) and +4 opaque (G) and transparent (H) phase variant strains which were prepared using a ruthenium red and lysine acetate with LR Resin protocol.

Table 5.1 Analysis of capsule thickness of 10 random bacteria per TIGR4(-) capsular switch strains as determined by measuring the total area within the cell wall, the area including capsule polysaccharide and calculating an average capsule thickness using the formula area = Πr^2. There is no statistical difference between the thickness of the capsules of the opaque (ANOVA $P > 0.05$) capsular switch strains.

TIGR4(-)+ Strain	**Opaque Phase Capsule Thickness (nm ± SD)**	**Transparent Phase Capsule Thickness (nm ± SD)**	**Opaque v Transparent *P*-value**
6A	137 ± 20	96 ± 15	$P < 0.05$
7F	148 ± 32	88 ± 10	$P < 0.001$
23F	142 ± 23	94 ± 9	$P < 0.01$
4	152 ± 26	86 ± 13	$P < 0.001$

5.2.2 Effect of capsular serotype on C3b/iC3b deposition on *S. pneumoniae* in human serum

To investigate the effect of capsular serotype on complement, C3b/iC3b deposition was investigated using flow cytometry on TIGR4 *S. pneumoniae* opaque phase variant strains. C3b/iC3b deposition was dependent on capsular serotype, with increased C3b/iC3b deposition on the TIGR4(-)+23F and TIGR4(-)+6A strains compared to the TIGR4(-)+4 and +7F strains ($P < 0.001$, Fig 5.3). Results for C3b/iC3b deposition on the TIGR4 and TIGR4(-)+4 strains in 100% human sera were similar (FI of 5000 ± 1940 versus 3910 ± 450 respectively, Fig 3.3A), demonstrating that the process of creating the mutant capsular switch strains did not have a significant affect C3b/iC3b deposition on *S. pneumoniae*. In addition, C3b/iC3b deposition on the opaque capsular switch strains was compared to deposition on the same strains in transparent phase. The pattern of C3b/iC3b deposition on the transparent strains was the same as on the opaque phase strains, with a significant increase on the TIGR4(-)+23F and TIGR4(-)+6A strains ($P< 0.001$, Fig. 5.4). Furthermore, C3b/iC3b deposition was increased on each strain in transparent phase compared to the same strain in opaque phase, indicating that the thickness of the polysaccharide capsule affects C3b/iC3b deposition.

Immunogold EM was used to identify the quantity and site of C3b/iC3b bound to opaque TIGR4 capsular switch strains. In agreement with the flow cytometry results on the opaque strains, the TIGR4(-)+6A and +23F strains had a median of 29.0 and 22.5 gold particles per bacterium respectively compared to 5.5 and 5.0 gold particles per bacterium for the TIGR4(-)+4 and +7F strains respectively (Fig. 5.5 and 5.6). The increased number of gold particles on the TIGR4(-)+6A and +23F strains were concentrated in clusters of up to 26

gold particles at one point on the bacterial surface (Fig. 5.6), whereas on the TIGR4(-)+4 and +7F there were no more than 3 gold particles at a given point on the bacterial surface. These observations suggest on the TIGR4(-)+6A and +23F there has been greater focal amplification of C3b/iC3b deposition, which is known to be dependent on the alternative complement pathway (Walport 2001; Brown et al. 2002). This pattern was also found to be true for the transparent strains, with the +6A and +23F strains having an increased number of immunogold per bacterium compared to the +7F or +4 strains (Fig 5.7). Furthermore, there was increased immunogold staining on each transparent phase strain compared to the same capsular switch strain in opaque phase, also confirming the results of the flow cytometry assay (Fig 5.5) and in accordance with results presented in Chapter 3.

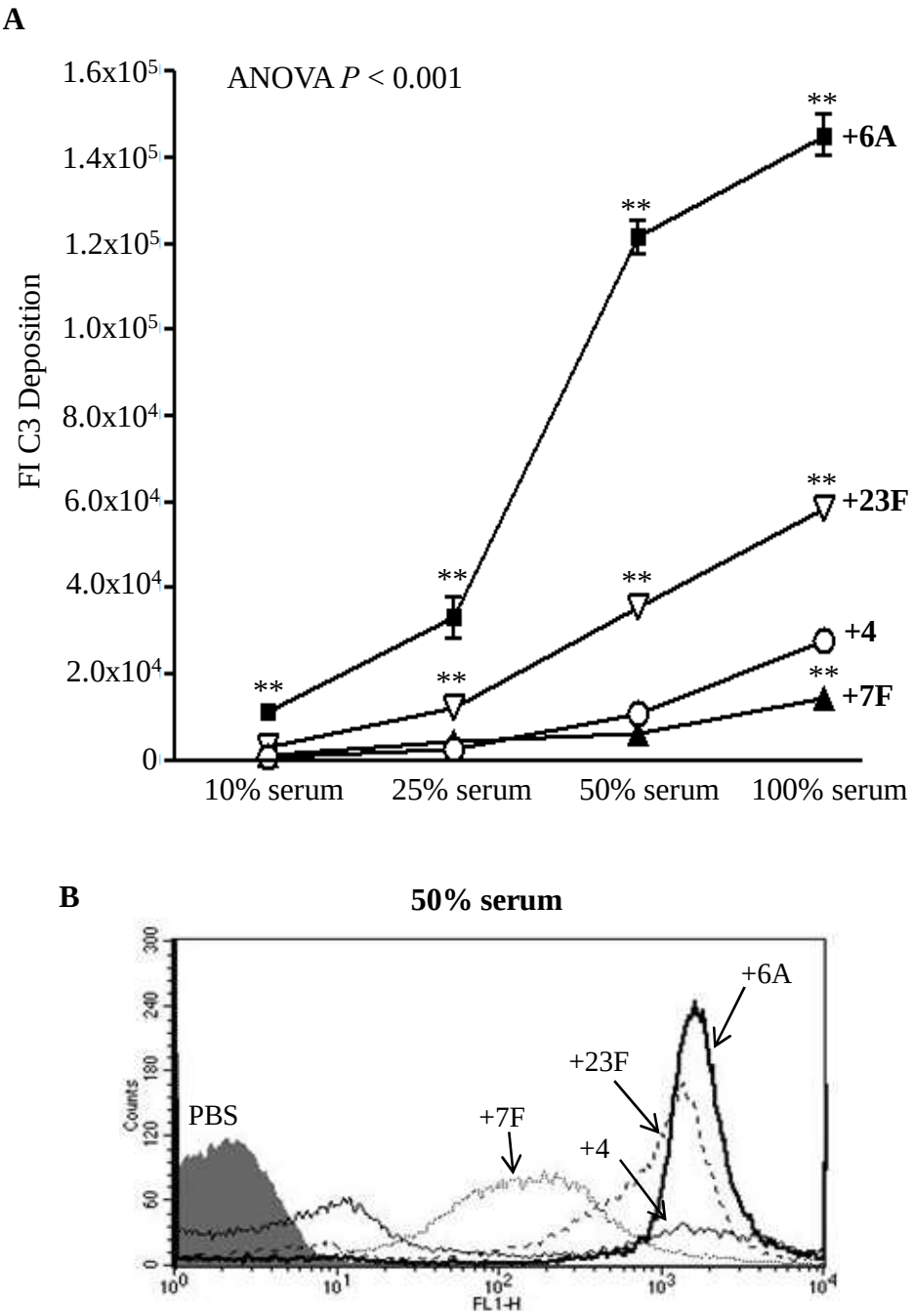


Fig 5.3 C3b/iC3b deposition on opaque capsular switch strains in human serum
(A) FI of C3b/iC3b deposition measured using flow cytometry on capsular switched TIGR4(-)+ opaque phase strains expressing capsular serotypes 6A (■), 7F (▲), 23F (▽) and 4 (○) in increasing concentrations of human serum. (B) Examples of flow cytometry histograms for C3b/iC3b deposition on TIGR4(-)+ capsular switched strains in 50% human serum. Grey shadowing indicates the results for bacteria incubated in PBS alone. Error bars represent SDs, * $P < 0.01$, ** $P < 0.001$ (ANOVA with post-hoc tests).

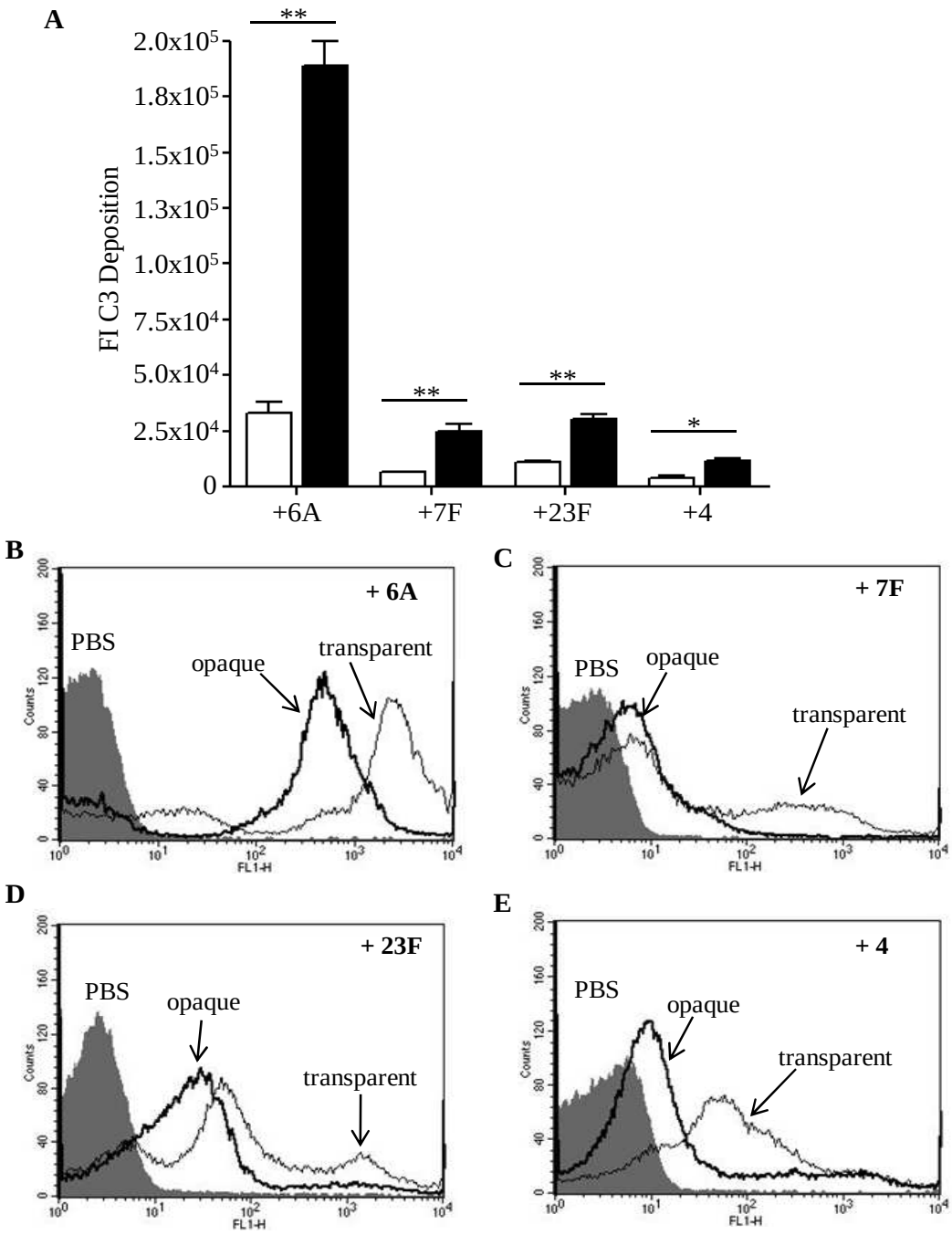


Fig 5.4 C3b/iC3b deposition on TIGR4 capsular switch strains in human serum
(A) FI of C3b/iC3b deposition measured using flow cytometry on capsular switched TIGR4(-)+ strains expressing capsular serotypes 6A, 7F, 23F and 4 in opaque (open bars) and transparent (closed bars) phase in 25% human serum. Error bars represent SDs and **P* < 0.01 or ***P* < 0.001 compared to the TIGR4(-)+4 strain (ANOVA with post hoc tests). (B), (C), (D), (E) Examples of flow cytometry histograms for C3b/iC3b deposition in 25% serum on TIGR4(-) + 6A (B), 7F (C), 23F (D) and 4 (E) opaque (thick lines) and transparent (thin lines) strains.

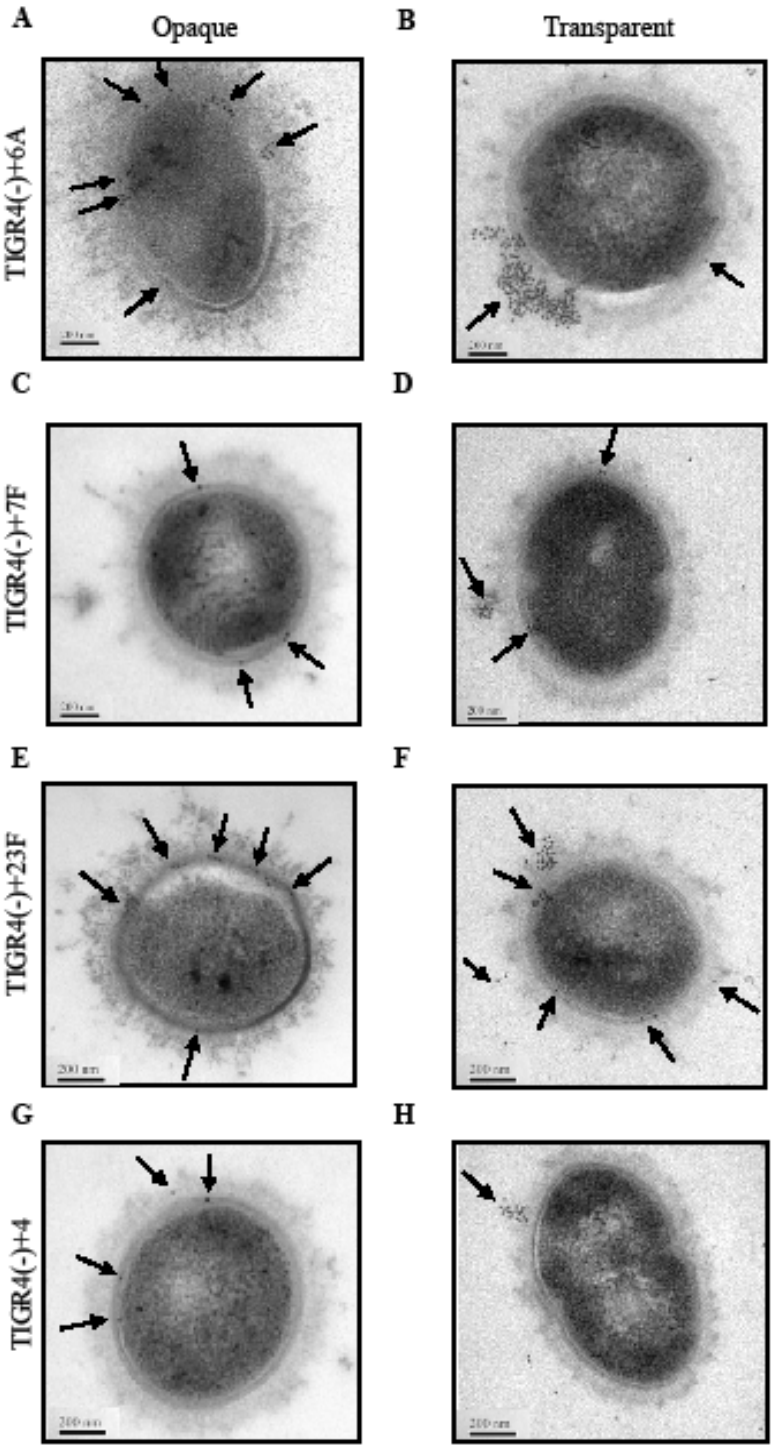
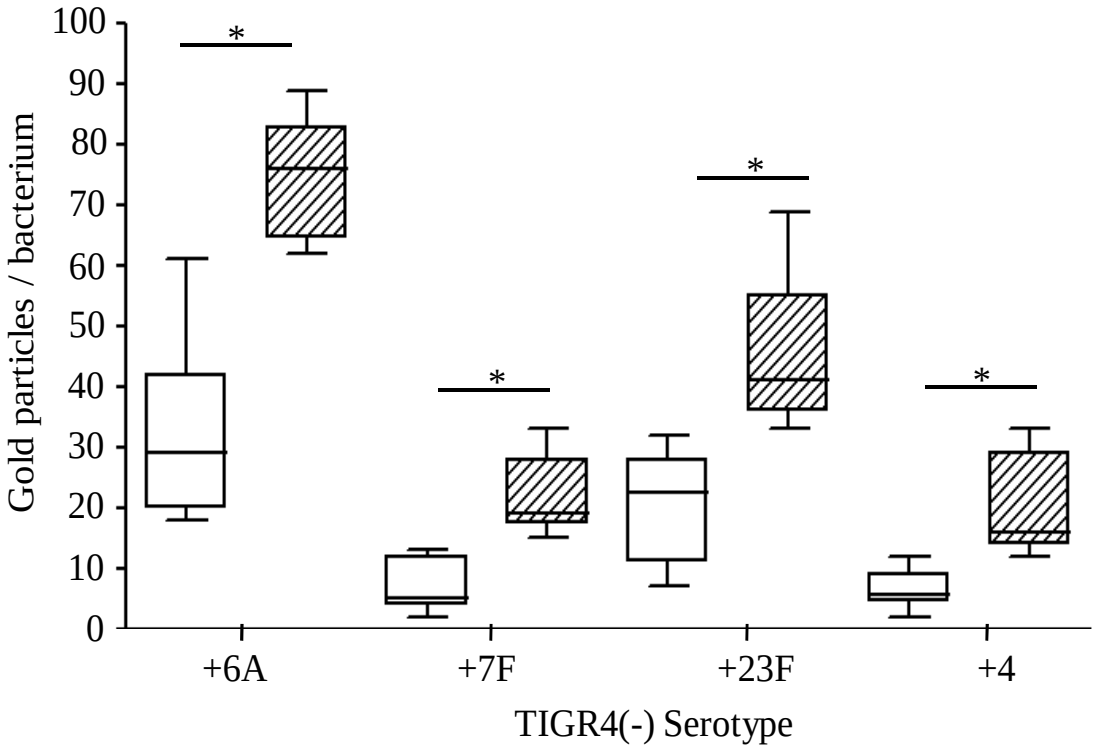


Fig 5.5 Immunogold against C3b/iC3b on TIGR4 capsular switch strains
(A) to (H) Representative immunogold EM images of C3b/iC3b deposition on the TIGR4(-) +6A opaque (A) and transparent (B), +7F opaque (C) and transparent (D), +23F opaque (E) and transparent (F) and +4 opaque (G) and transparent (H) strains.



<u>Fig 5.6</u> Quantitation of immunogold against C3b/iC3b on TIGR4 capsular switch strains

Number of C3b/iC3b-labelled gold particles identified by immunogold EM deposited per bacterium for TIGR4(-)+ capsular switched strains (medians and interquartile range (IQR), n=10 per strain) in opaque (open bars) or transparent (slashed bars). *P < 0.05 (Kruskal Wallis with Dunn's multiple comparison test) compared to the TIGR4(-)+4 strain. For comparisons between the capsular switch strains in opaque phase, the serotype +6A and +23F were significantly different from the TIGR4(-) +4 strain (P < 0.01 Kruskal Wallis with Dunn's multiple comparison test).

5.2.3 The effect of capsular serotype on alternative and classical pathway activity

To provide further evidence that differences in C3b/iC3b deposition on the opaque TIGR4(-)+ strains were dependent on the alternative pathway, the C3b/iC3b flow cytometry assays were repeated in sera depleted in specific components of the alternative (Bf^-) or classical ($C1q^-$) pathways, using $C9^-$ serum as a control (Yuste et al. 2006; Yuste et al. 2008). As previously shown (Yuste et al. 2008) C3b/iC3b deposition was markedly reduced on all the TIGR4(-)+ strains in $C1q^-$ and Bf^- sera (Fig. 5.7 A, C and E). The patterns of C3b/iC3b deposition on the TIGR4(-)+strains in $C1q^-$ and $C9^-$ serum were similar to that obtained for normal human sera, with increased C3b/iC3b deposition on the TIGR4(-)+6A strain and smaller increases on the +23F strain. However, in Bf^- serum there were no differences in C3b/iC3b deposition between the TIGR4(-)+23F and +4 or +7F strains, and the increased in C3b/iC3b deposition on the +6A strains was proportionally less than that seen with $C1q^-$ or $C9^-$ sera.

The classical pathway can be activated through either innate or adaptive immune mechanisms, which may be differentially affected by the varying structures of the capsule polysaccharides. Differences in the binding of the classical complement pathway mediators C1q, CRP and SAP to the capsular switch strains were investigated using flow cytometry. There was increased binding of both C1q and CRP in the transparent strains, indicating that the thickness of the capsule affects binding of these complement mediators (Fig. 5.8 A and B). However, in contrast to the C3b/iC3b results, both the TIGR4(-) +6A and +7F strains showed high levels of C1q binding and there was no statistically significant difference in C1q binding between the TIGR4(-) +23F and +4 strain (ANOVA $P > 0.05$, Fig. 5.8 A). CRP binding was significantly reduced on the TIGR4(-) +7F strain (ANOVA $P<0.05$) but

there was no other significant difference in the relative binding of this complement mediator to the other capsular switch strains (Fig. 5.8 D). In line with the results from TIGR4, TIGR4*cps*, D39 and D39-DΔ strains (Fig 3.13 A), there was increased SAP on the opaque phase variants compared to strains in transparent phase (Fig. 5.10 A). There was decreased SAP binding on the +6A and +23F strains compared to the +7F and +4 capsular switch strains (ANOVA with post-hoc tests $P < 0.01$). There was no correlation between the C3b/iC3b deposition results and binding of the classical pathway mediators C1q, SAP and CRP (Szalai et al. 1996; Brown et al. 2002; Yuste et al. 2007) to the TIGR4(-)+ strains.

The activity of the alternative pathway is inhibited by the regulatory protein FH, which is known to interact with CbpA. Whilst CbpA expression is increased on the transparent variants, there may also be increased FH access to CbpA due to the thinner CPS. The transparent phase strains behaved in a similar manner to the unencapsulated strains (Fig 3.13 B), with more FH binding compared to the opaque phase TIGR4 strains. However, there was no statistical difference in FH deposition between the 6A, 7F and 23F expressing strains, with a lower amount of FH deposited on the TIGR4(-) +4 strain (Fig. 5.10 D), indicating that increased FH binding was not accounting for the relatively low C3b/iC3b deposition on the TIGR4(-) +7F and +4 strains. Overall, these data suggest that the type of capsular serotype expressed by the TIGR4(-)+ strains has a marked effect on C3b/iC3b deposition that was largely dependent on alternative rather than classical complement pathway activity.

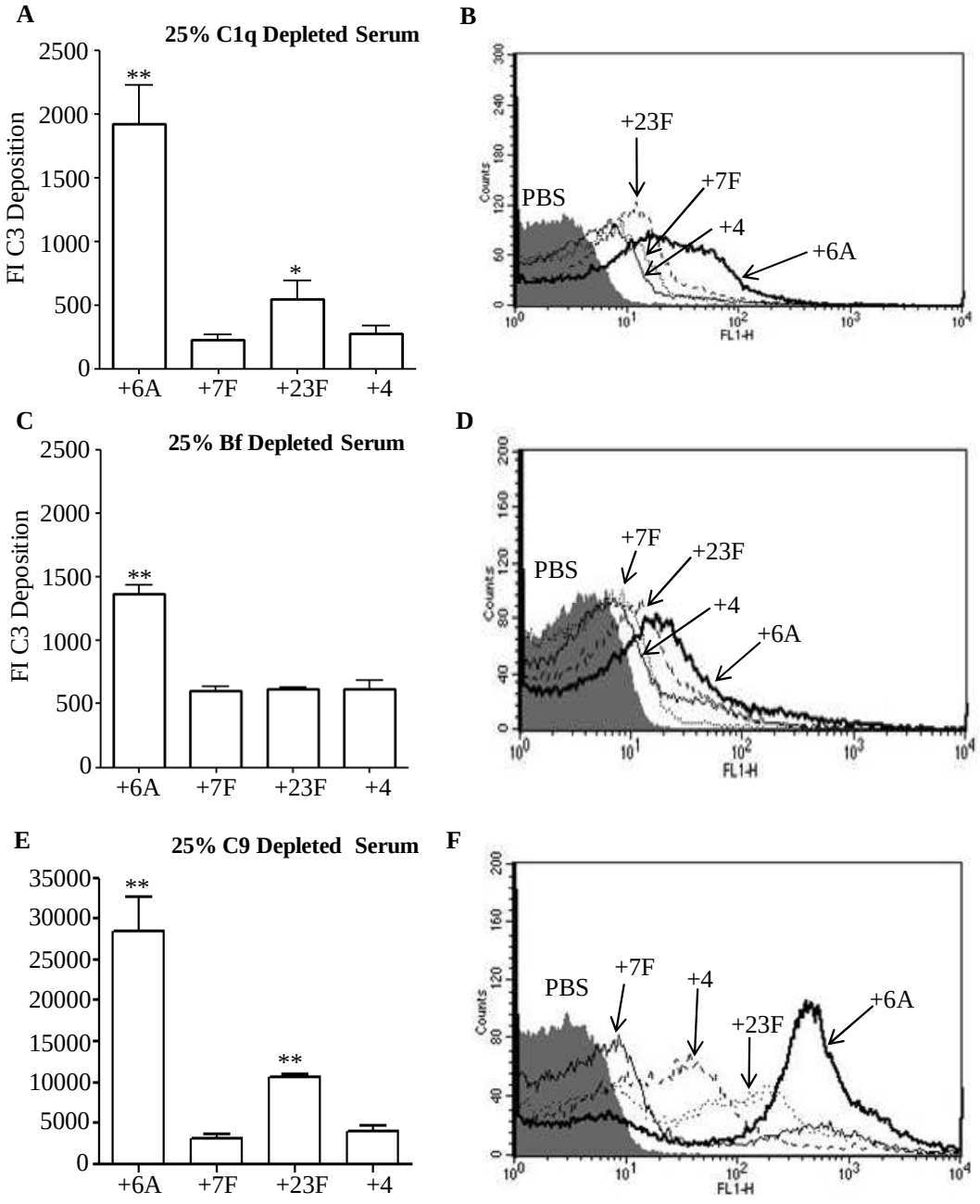
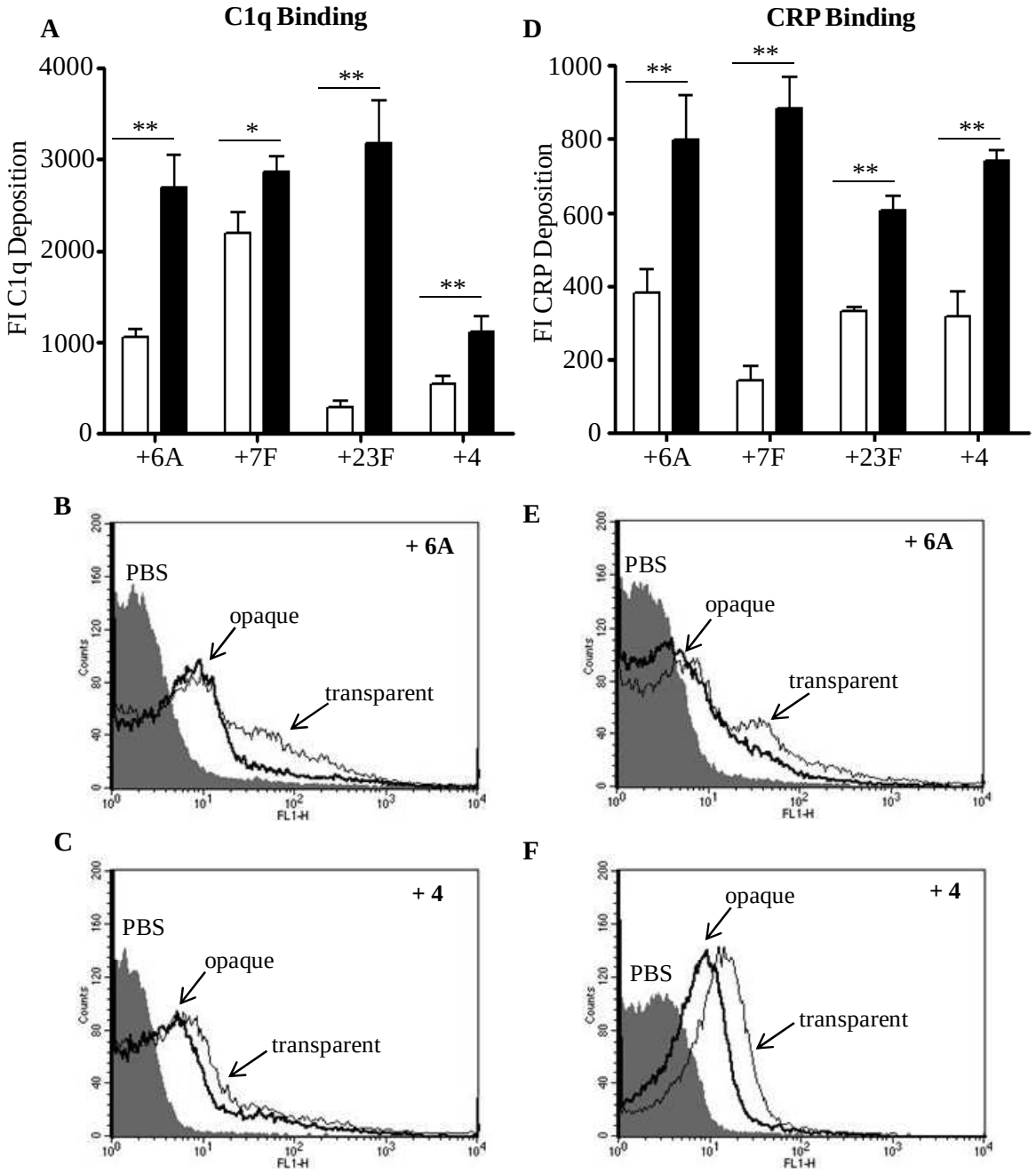


Fig 5.6 C3b/iC3b deposition on opaque TIGR4 capsular switch strains in complement depleted human serum

(A), (C), (E). FI of C3b/iC3b deposition measured using flow cytometry on the opaque phase TIGR4(-)+ capsular switched strains in 25% human serum depleted of complement component C1q (A), factor B (C) or C9 (E). Error bars represent SDs and **P* < 0.01 or ***P* < 0.001 compared to the TIGR4(-)+4 strain (ANOVA with post hoc tests). (B), (D), (F) Examples of flow cytometry histograms for C3b/iC3b deposition on TIGR4(-)+ 6A (thick black line), 7F (dotted line), 23F (dashed line) or 4 (thin solid line) strains in 25% C1q depleted (B), factor B depleted (D) or C9 depleted (F) human serum.



<u>Fig 5.8</u> C1q and CRP binding to the TIGR4 capsular switch strains

(A) C1q deposition on TIGR4(-) capsular switch strains in opaque (open bars) and transparent (solid bars) phase in 25% human serum. (B), (C) Representative flow cytometry histograms of C1q deposition on opaque (thick line) and transparent (thin line) TIGR4(-) + 6A and +4 respectively. (D) CRP deposition on TIGR4(-) capsular switch strains in opaque (open bars) and transparent (solid bars) phase in 25% human serum. (E), (F) Representative flow cytometry histograms of CRP deposition on opaque (thick line) and transparent (thin line) TIGR4(-) + 6A and +4 respectively. For (A) and (D) error bars represent SDs and *P < 0.01 or **P < 0.001 (ANOVA with post hoc tests).

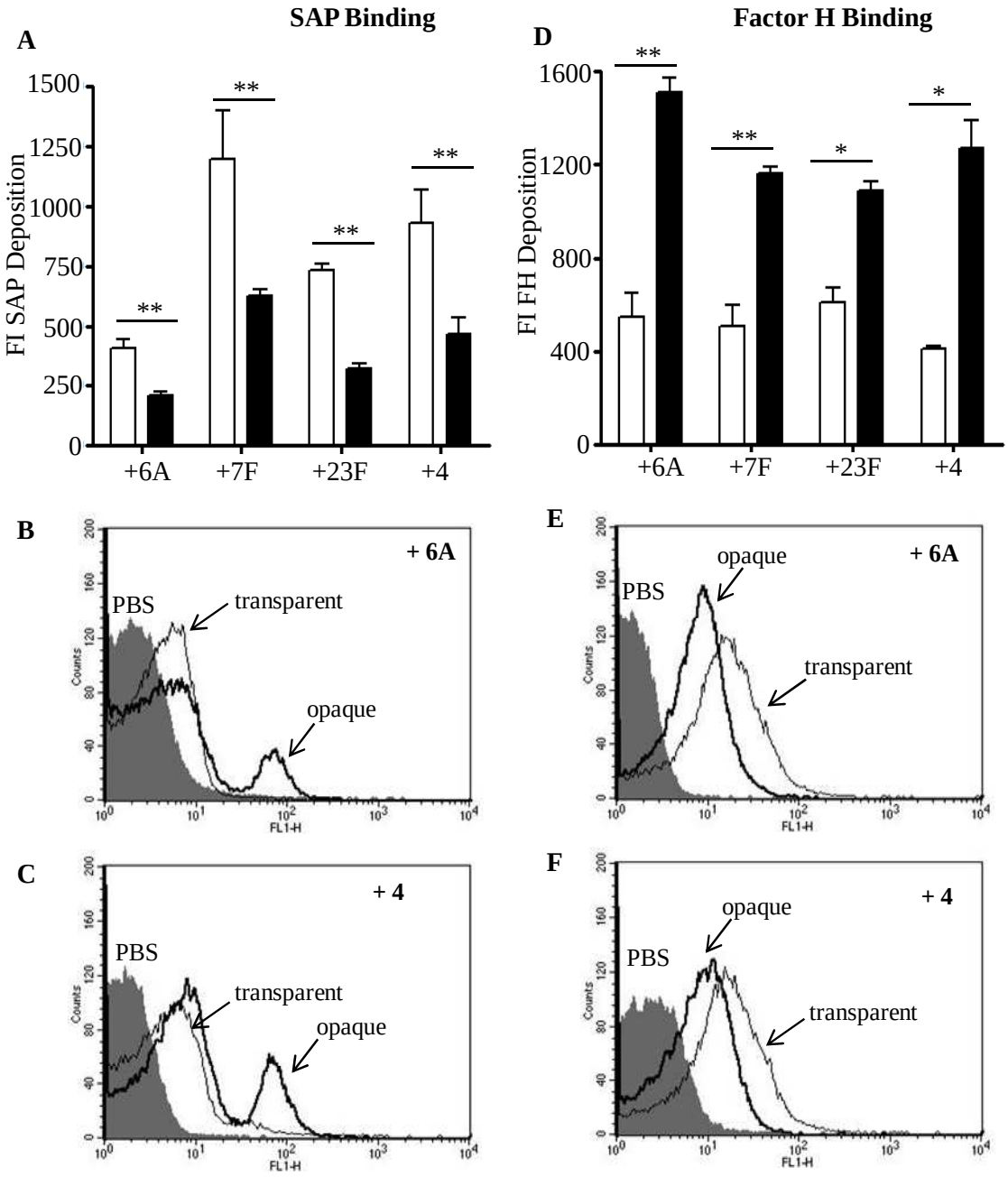


Fig 5.9 SAP and FH binding to the TIGR4 capsular switch strains

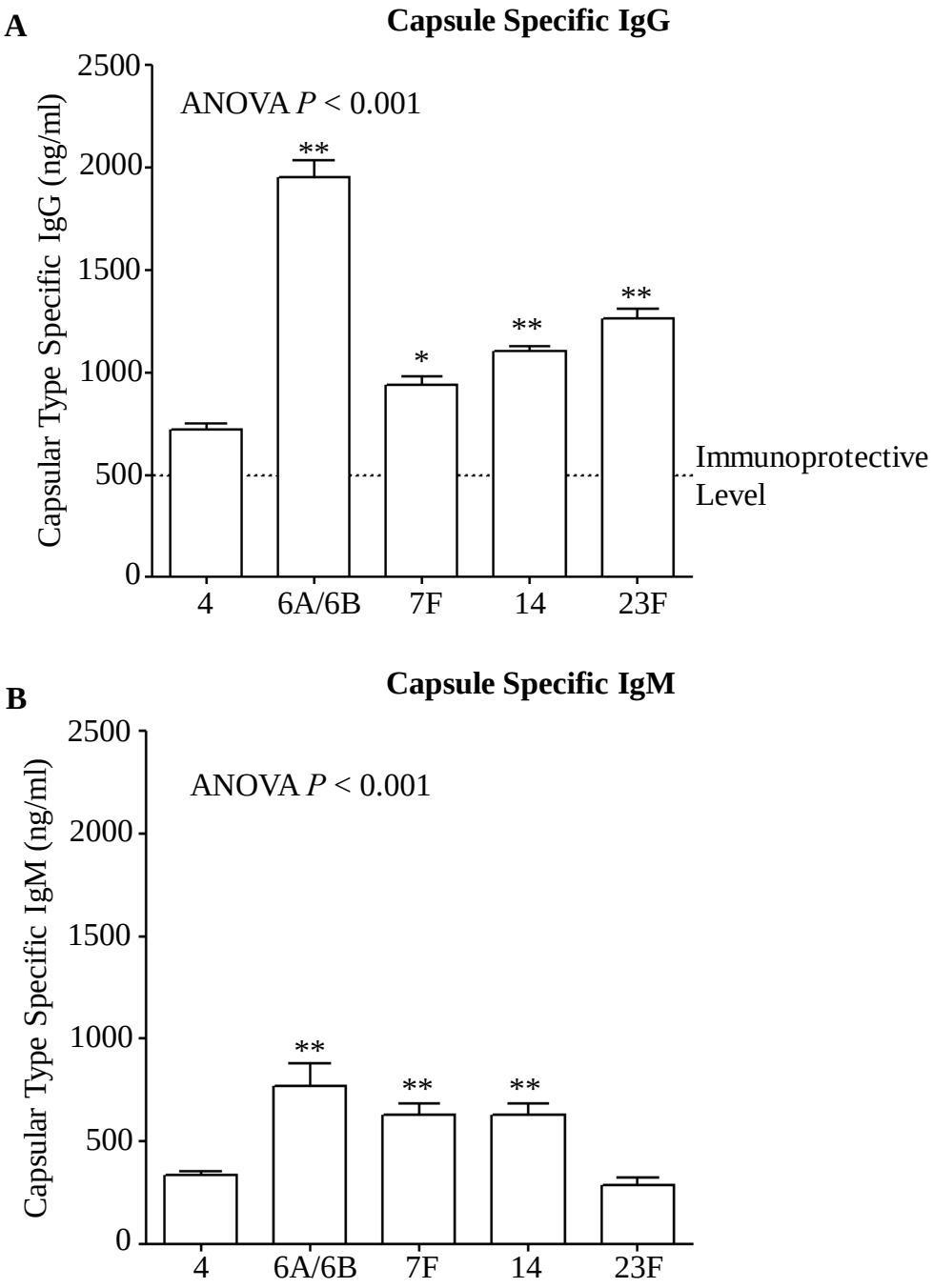
(A) SAP deposition on TIGR4(-) capsular switch strains in opaque (open bars) and transparent (solid bars) phase in 25% human serum. (B), (C) Representative flow cytometry histograms of SAP deposition on opaque (thick line) and transparent (thin line) TIGR4(-) + 6A and +4 respectively. (D) Factor H deposition on TIGR4(-) capsular switch strains in opaque (open bars) and transparent (solid bars) phase in 25% human serum. (E), (F) Representative flow cytometry histograms of factor H deposition on opaque (thick line) and transparent (thin line) TIGR4(-) + 6A and +4 respectively. For (A) and (D) error bars represent SDs and $*P < 0.01$ or $**P < 0.001$ (ANOVA with post hoc tests).

5.2.4 The effect of antibody on C3b/iC3b deposition

Since antibody can activate the classical complement pathway, the levels of capsular specific IgG and IgM against serotype 6A, 7F, 4, 23F and 14 was quantitated using the WHO ELISA protocol (Wernette et al. 2003) (Fig. 5.10 A and B). There was variation in the levels of both IgG and IgM in the human serum against the different capsular serotype. The IgG titres were found to correlate closely with C3b/iC3b deposition flow cytometry results for the TIGR4(-)+ strains (Pearson's R^2 of 0.98 with $P = 0.021$), with high antibody levels against serotype 6A/6B (the ELISA is unable to distinguish between serotype 6A and 6B). These data suggest the effects of capsular serotype on C3b/iC3b deposition could be due to differences in levels of capsule specific antibody. However, other than the polysaccharide capsule, *S. pneumoniae* has multiple antibody generating antigens which are present on the cell surface. Different capsule polysaccharides could affect the ability of these antibodies to bind to sub-capsular targets and therefore a flow cytometry assay which detects the relative total IgG and IgM binding to each TIGR4 capsular switch strain was performed. Results for total IgG or IgM binding to the +6A and +4 strain were not statistically significant (ANOVA $P > 0.05$, Fig. 5.11 A and B), and overall there was no correlation between flow cytometry results for C3b/iC3b deposition and total IgG binding to the TIGR4(-)+ strains in human serum (Pearson's $R^2 < 0.0001$, $P = 1.0$). To clarify the importance of antibody for the differences in C3b/iC3b deposition on the TIGR4(-)+ strains, C3b/iC3b assays were repeated using baby rabbit complement and mouse serum, both of which have no detectable IgG binding or capsular specific antibodies to *S. pneumoniae* (Yuste et al. 2008). As expected the absolute results for these sera were different to those for human sera due to differences in complement activity and the species-specific anti-C3 antibodies used. However, the pattern of C3b/iC3b deposition on the

TIGR4(-)+ strains in baby rabbit complement and mouse serum was similar to that obtained with normal human serum, with large increases on the TIGR4(-)+6A strain and (in baby rabbit complement) a less marked increase on the TIGR4(-)+23F strain, compared to the TIGR4(-)+4 and +7F strains (Fig. 6.12 A and C). Hence the differences in C3b/iC3b deposition between the TIGR4(-)+ capsular switched strains may reflect an inherent property of capsular serotype.

The pattern of C3b/iC3b deposition in $C1q^-$ human serum (in which antibody mediated complement activity is absent) on TIGR4(-)+ strains was similar to that obtained with complement sufficient serum (Fig. 5.7 A). Furthermore, repeated C3b/iC3b flow cytometry assays using test human serum which was depleted of IgG using IdeS demonstrated that in there was still increased C3b/iC3b deposition on the TIGR4(-)+6A and +23F strains relative to the TIGR4(-)+4 and +7F strains when the effect of antibody was removed from human serum (Table 5.2). Overall, these results suggest that the differences between C3b/iC3b deposition on the TIGR4 capsular switch strains cannot be accounted for by differing levels of capsular specific antibody present in the serum.



<u>Fig 5.10</u> Quantitation of capsular serotype specific IgG and IgM in human serum
(A), (B) Quantitation using ELISA of levels of capsular serotype specific IgG (A) and IgM (B) in WT serum used in experiments throughout this thesis. For all panels, error bars represent SDs and **P* < 0.01 or ***P* < 0.001 compared to the TIGR4 WT strain (ANOVA with post hoc tests).

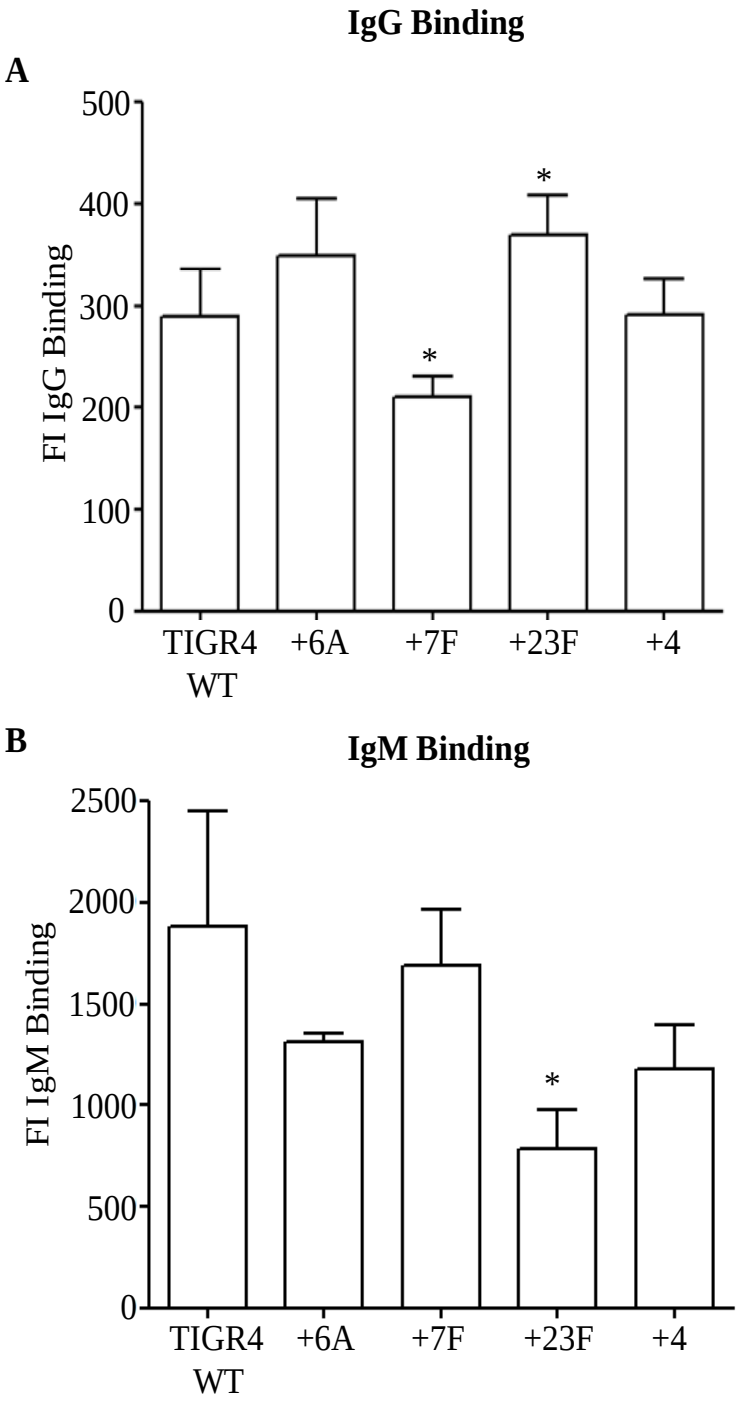
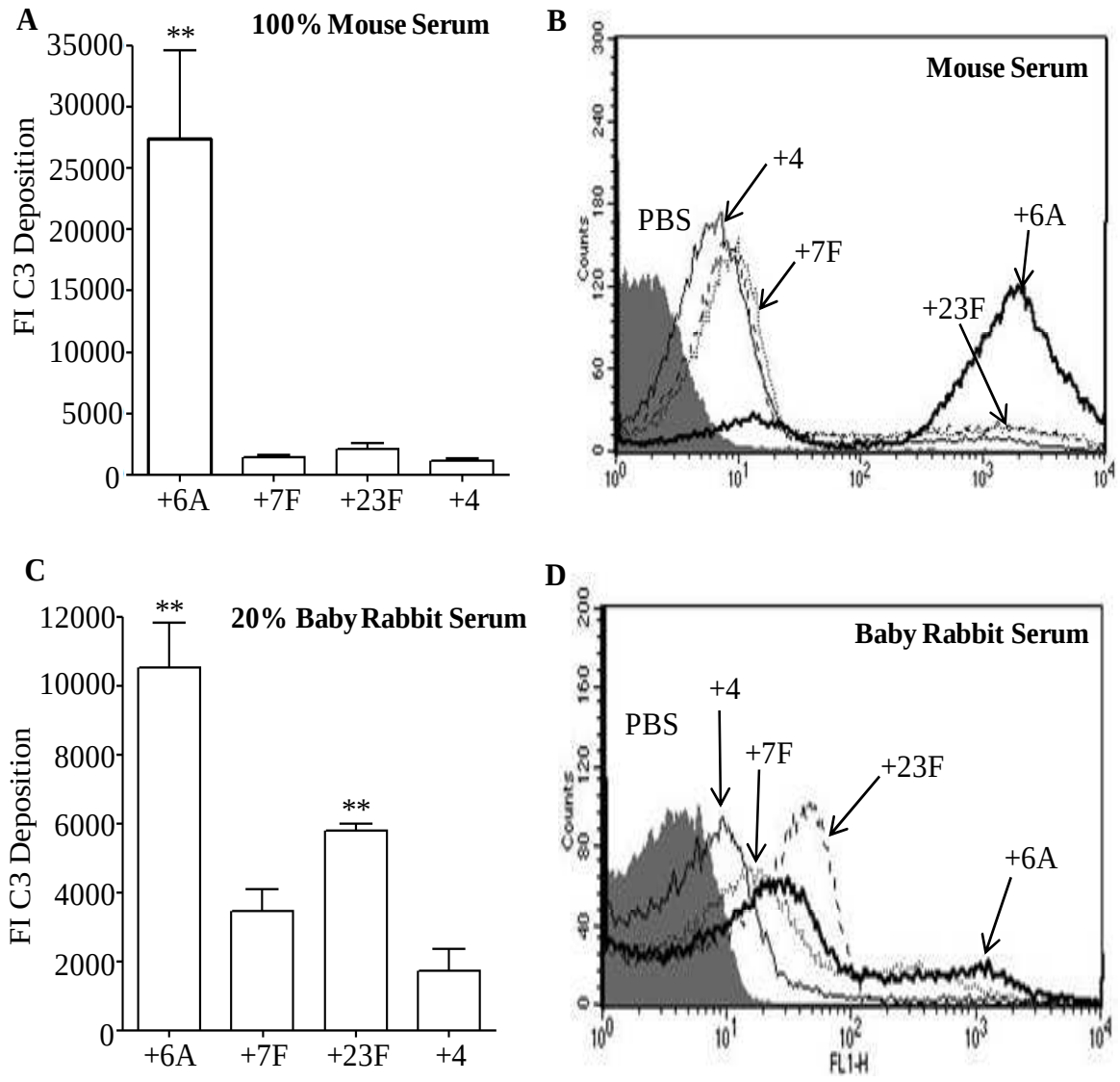


Fig 5.11 IgG and IgM binding to opaque capsular switch strains
(A), (B) FI of IgG (A) or IgM (B) deposition measured using flow cytometry on capsular switched TIGR4(-)+ opaque phase strains expressing capsular serotypes 6A, 7F, 23F and 4 in 25% human serum. For both panels, error bars represent SDs and *$P < 0.05$ compared to the TIGR4 WT strain (ANOVA with post hoc tests).



<u>Fig 5.12</u> C3b/iC3b on opaque capsular switch strains opsonised in mouse or rabbit serum

(A), (C) FI of C3b/iC3b deposition measured using flow cytometry on the TIGR4(-)+ capsular switched strains in 100% mouse serum (A) or 20% baby rabbit serum (C). Error bars represent SDs and $*P < 0.01$ or $**P < 0.001$ compared to the TIGR4(-)+4 strain (ANOVA with post hoc tests). (B), (D) Examples of flow cytometry histograms for C3b/iC3b deposition on TIGR4(-)+ 6A (thick black line), 7F (dotted line), 23F (dashed line) or 4 (thin solid line) strains in 100% mouse (B) or 20% baby rabbit serum (D).

Table 5.2 Effects of IgG depletion using IdeS on mean FI +/- SDs of C3b/iC3b deposition and C1q binding to opaque TIGR4 capsular switch strains in 25% human serum. *P* values represent comparisons between the results for TIGR4(-)+6A, 7F and 23F against the TIGR4(-)+4 strain using ANOVA with Dunnett's multiple comparison.

TIGR4(-) Strain	**C3b/iC3b deposition (FI ± SD) BSA treated**	***P* value**	**C3b/iC3b deposition (FI ± SD) IdeS treated**	***P* value**
+6A	55240 ± 7400	< 0.01	22440 ± 1670	< 0.01
+7F	3930 ± 860	> 0.05	1200 ± 250	> 0.05
+23F	16250 ± 450	< 0.01	4000 ± 170	< 0.01
+4	5520 ± 620	--	1650 ± 200	--

5.2.5 The level of C3b/iC3b deposition on capsular switch strains correlates with neutrophil phagocytosis

The functional consequences of differences in C3b/iC3b deposition on the opaque TIGR4(-)+ strains were investigated using a flow cytometry neutrophil phagocytosis assay. This assay identifies the proportion of neutrophils associated with fluorescent bacteria, which data acquired using cytochalasin D suggests mainly represents internalisation of *S. pneumoniae* (Yuste et al. 2008). Bacteria were opsonised using mouse serum or baby rabbit complement to remove any complement independent antibody effects since levels varied against the different TIGR4(-)+ strains in test human serum. As expected phagocytosis of all strains was complement dependent, increasing with higher concentrations of serum, and with similar levels for bacteria incubated in heat treated (which inactivates complement) serum (Yuste et al. 2008) or buffer alone (Fig. 5.13 A and B). In buffer or heat inactivated serum there were no significant differences between the TIGR4(-)+ strains, but in both mouse serum and baby rabbit complement the TIGR4(-)+6A and to a lesser extent the TIGR4(-)+23F strains were more susceptible to phagocytosis than the TIGR4(-)+4 and +7F strains (Fig. 5.13 A and B). There was a positive correlation for the results of phagocytosis with C3b/iC3b deposition measured by flow cytometry in 20% mouse serum (Pearson's R^2 0.93, P = 0.035) and in 25% baby rabbit complement (Pearson's R^2 0.93, P = 0.029). These data suggest the effects of capsular serotype on C3b/iC3b deposition lead to differences in phagocytosis.

To demonstrate the effect of antibody and complement on opsonophagocytosis in human serum, assays were repeated using test human serum which had been treated with IdeS to cleave the Fc fragment of the IgG. There was reduced neutrophil phagocytosis of all of the

capsular switch TIGR4 strains in IdeS treated serum compared to the control (BSA treated) human serum, and both the TIGR4(-)+6A and +23F strains showed increased association with neutrophils compared to the TIGR4(-)+7F and +4 strains. However when the opaque capsular switch strains were opsonised with serum in which complement was inactivated by heat treatment there was no difference between the different capsular serotypes (Table 5.3). In contrast to opsonisation with mouse and rabbit serum, the TIGR4(-) +7F showed the greatest association with neutrophils in control serum compared to the other TIGR4 capsular switch strains, indicating a significant effect of anti-capsular antibody on neutrophil phagocytosis against this serotype (Table 5.3).

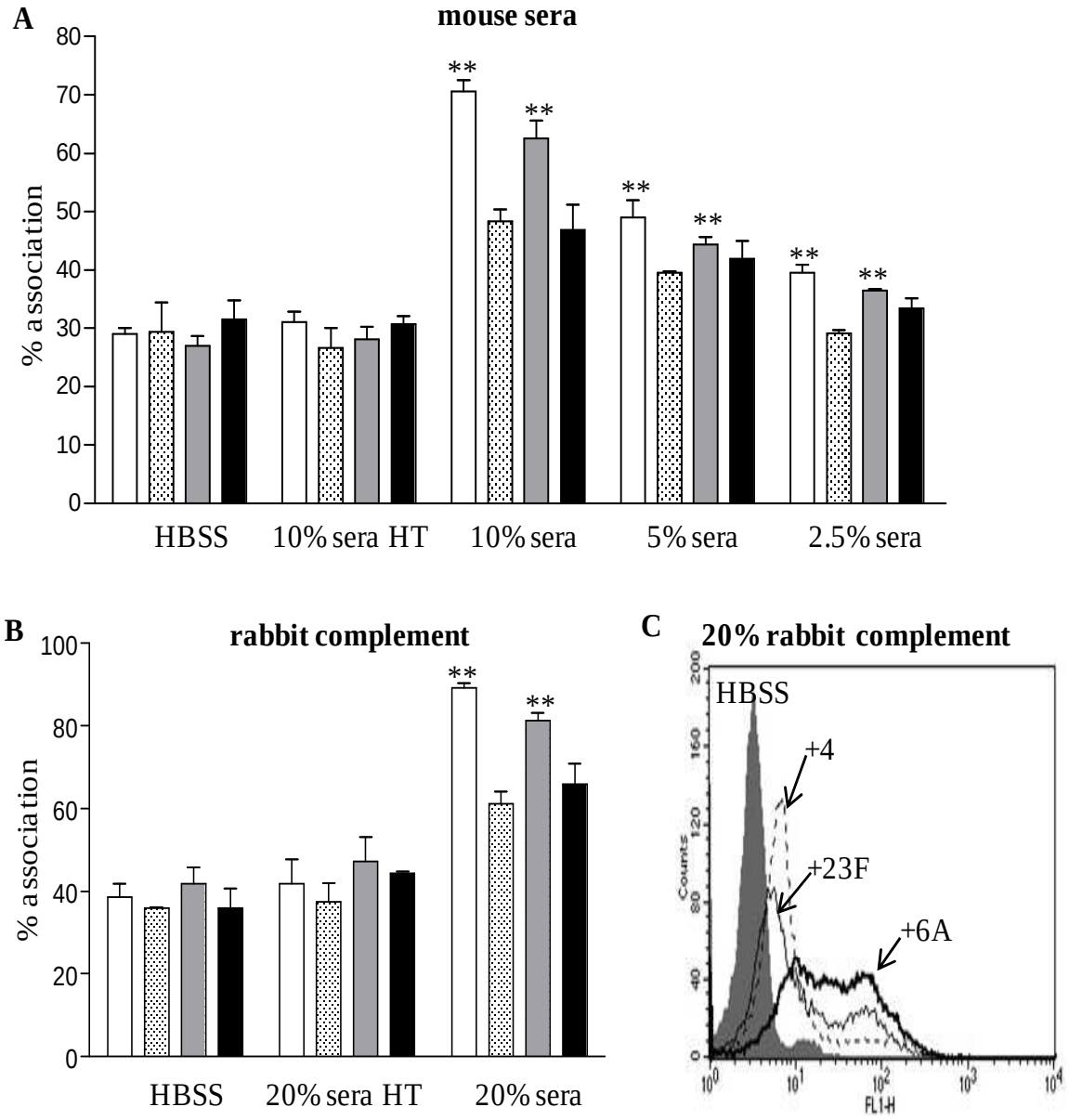


Fig 5.13 Neutrophil phagocytosis of TIGR4 capsular switch strains

(A), (B) Neutrophil phagocytosis measured using flow cytometry of TIGR4(-)+6A (white columns), +7F (stippled columns), +23F (grey columns) or +4 (black columns) strains after incubation in HBSS buffer, heat treated (HT) 10% mouse serum, or 10%, 5% or 2.5% mouse serum (A), or heat treated (HT) 20% baby rabbit complement or 20% baby rabbit complement (B). Results are expressed as the mean (SD) proportion of neutrophils associated with fluorescent bacteria (labelled with FAMSE), $^{**}P < 0.001$ (ANOVAs with post-hoc tests) compared to the TIGR4(-)+4 strain. (C) Examples of flow cytometry histograms for the association of TIGR4(-)+4, +6A and +23F capsular switched strains with neutrophils after opsonisation with 20% baby rabbit complement. Grey shadowing indicates the results for bacteria incubated in HBSS alone. The histogram for the TIGR4(-)+7F strain is not included for ease of interpretation of this Fig and because this strain had no significant differences to the TIGR4(-)+4 strain.

Table 5.3 Effects of IgG depletion using IdeS and complement depletion by heat treatment on mean +/- SDs percentage association of TIGR4 capsular switch strains with neutrophils after incubation in HBSS, 20% normal human serum (NHS) or 20% IdeS (IdeS) or heat treated (HT) serum. * $P < 0.01$ for comparisons between the results for TIGR4(-)+6A, 7F and 23F against the TIGR4(-)+4 strain using ANOVA with Dunnett's multiple comparison.

TIGR4(-) Strain	HBSS	20% HT Serum	20% IdeS Serum	20% NHS
+6A	11.30 ± 1.25	20.50 ± 3.00	58.90 * ± 0.80	69.90 ± 1.30
+7F	11.60 ± 1.35	18.40 ± 1.00	42.10 ± 1.85	84.30 * ± 0.95
+23F	11.90 ± 2.60	19.00 ± 1.05	55.60 * ± 1.30	76.10 * ± 2.25
+4	11.30 ± 1.90	18.30 ± 1.10	44.70 ± 1.40	69.40 ± 1.26

5.2.6 The effect of capsular serotype on virulence in mouse models of infection

To determine if the effects of capsular serotype on complement deposition and phagocytosis were associated with differences in virulence a mouse model of septicaemia was used. Groups of five mice were infected by IP inoculation with 2000 CFU of the TIGR4(-)+ strains, and bacterial CFU recovered from blood and spleens after 24 hours calculated by plating serial dilutions (Fig. 5.14). In this model no unencapsulated TIGR4 strain are recovered from either spleen or blood at 24 hours (Chapter 3). For all the TIGR4(-)+ strains large numbers of bacteria were recovered from the blood and spleens after 24 hours, compatible with the known virulence of the TIGR4 strain in mice (Fig. 5.14) (Sandgren et al. 2005). The mean CFU in the spleens depended on capsular serotype, with significantly lower CFU recovered for the TIGR4(-)+6A and +23F strains (median CFU 3.40 and 5.87 x 10^6 respectively) compared to the TIGR4(-)+7F and +4 strains (median CFU 8.4 and 13.2 x 10^6 respectively) (Fig. 5.14 A). Similar results were obtained for the median CFU in blood (Fig. 5.14 B). Hence the TIGR4(-)+ strains with increased levels of C3b/iC3b deposition and neutrophil phagocytosis were less virulent in a mouse model of septicaemia.

Furthermore, we investigated the effects of capsular serotype in early lung infection by intranasally inoculating 5x10^6 CFU FAM-SE labelled TIGR4(-)+ strains, harvesting after 4 hours and performing bronchoalveolar lavages on CD1 outbred mice. The +6A and +23F strains showed significantly lower bacterial counts than the +4 and +7F capsular switch strains (Fig. 5.15 A), indicating that within the first 4 hours of lung infection their survival was already impaired. In addition, alveolar macrophages isolated by flow cytometry from BALF of mice inoculated with the +6A and +23F strains showed increased fluorescence,

suggesting that the decreased survival of these strains was associated with an increased uptake by resident alveolar macrophages (Fig. 5.15 B). TNFα levels in the BALF were significantly lower in mice inoculated with +6A and +23F strains compared to +7F and +4 strains (Fig. 5.16) showing that these capsular serotypes induced a lower inflammatory response in early lung infection which may be related to the lower CFU in BALF at this time point.

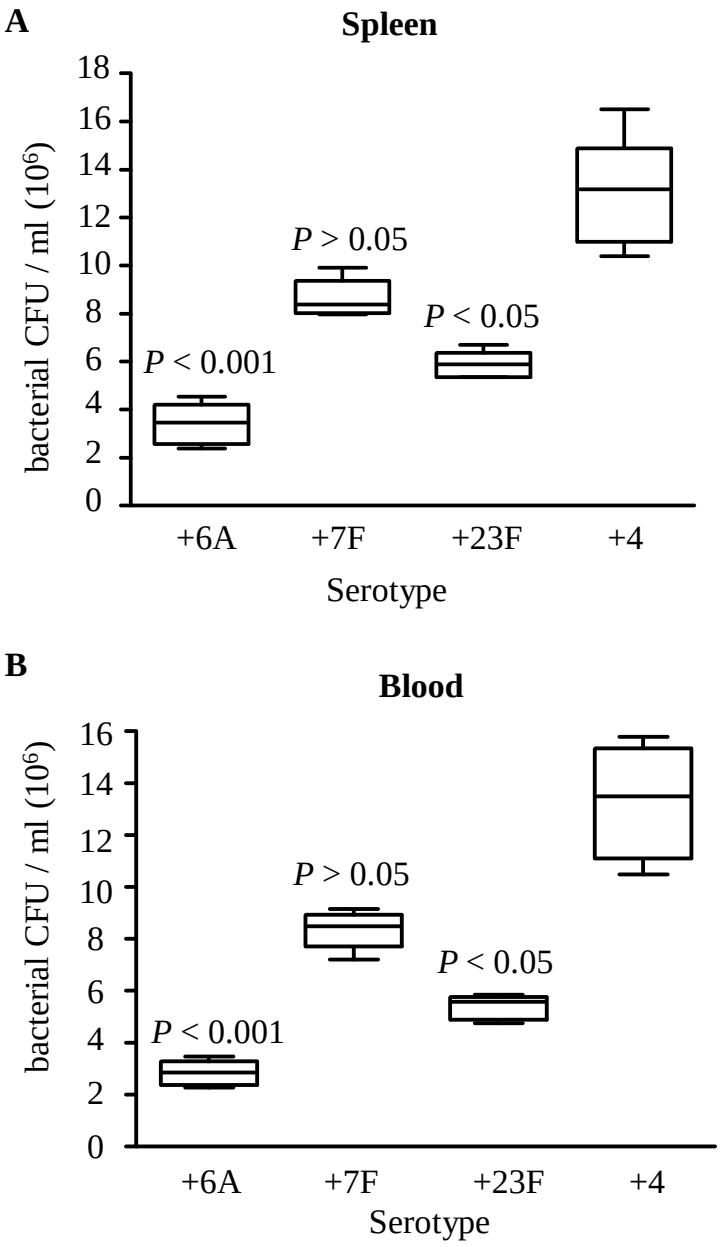
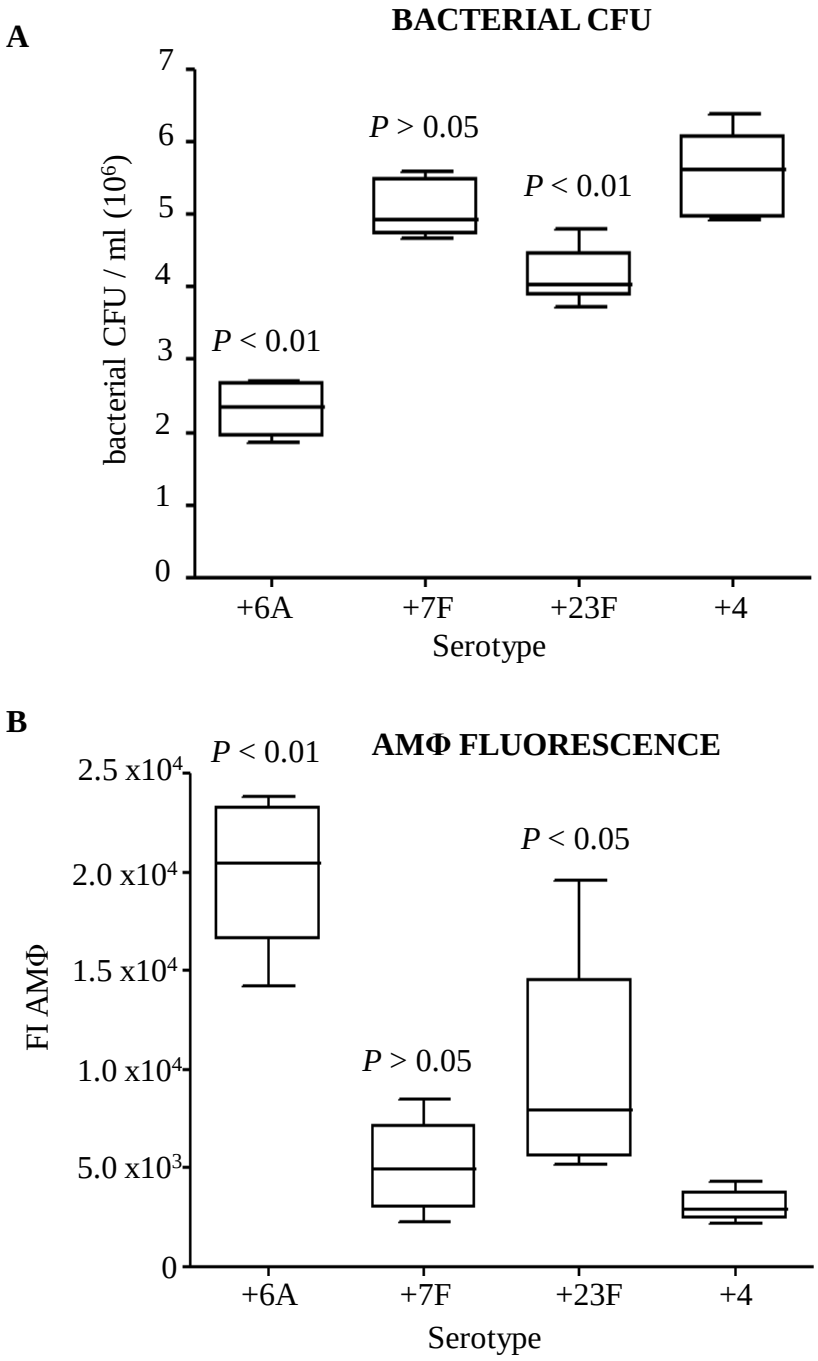


Fig 5.14 Virulence of the capsular switched TIGR4(-)+ strains in a septicaemia model (A), (B) Median (IQR) bacterial CFU recovered from mouse spleens (A) or blood (B) 24 hours after IP inoculation of 4000 CFU of the TIGR4(-)+6A, +7F, +23F or +4 strains. For both blood and spleen inoculation, $P = 0.0005$ (Kruskal-Wallis) for the overall comparison between strains. *P* values for individual strains compared to the TIGR4(-)+4 strain are given above the respective box and whisker plot (Dunn's multiple comparison test).



<u>Fig 5.15</u> Virulence of the capsular switched TIGR4(-)+ strains in early lung infection
(A) Median (IQR) bacterial CFU recovered from BALF 4 hours after IN inoculation of $5x10^6$ CFU of the TIGR4(-)+6A, +7F, +23F or +4 strains. (B) FI of alveolar macrophages following 4 hours IN inoculation. For both panels, P = 0.001 (Kruskal-Wallis) for the overall comparison between strains. P values for individual strains compared to the TIGR4(-)+4 strain are given above the respective box and whisker plot (Dunn's multiple comparison test).

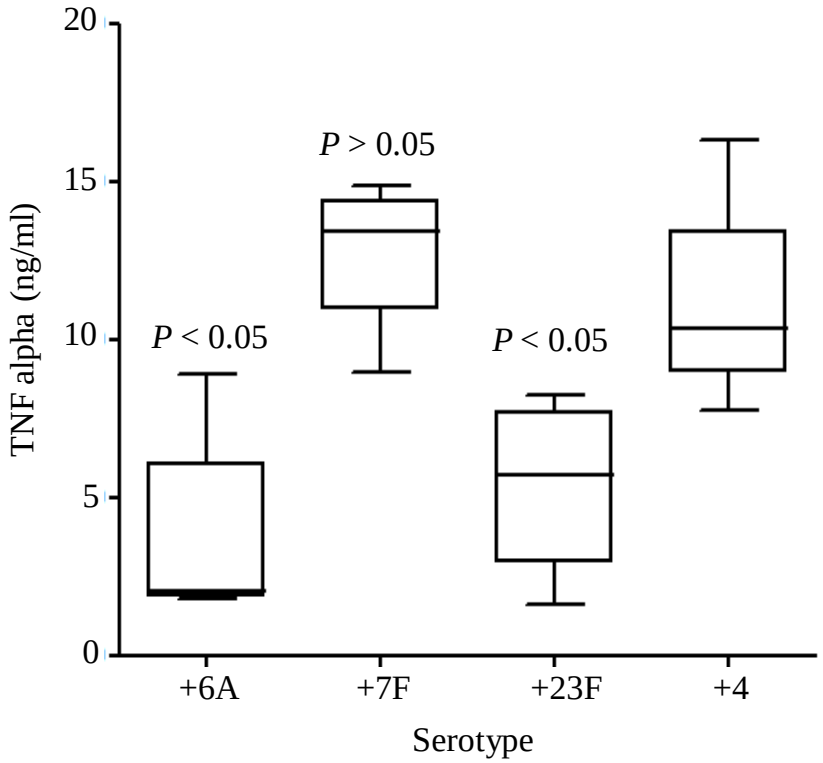


Fig 5.16 BALF TNFα levels 4hr after IN inoculation of TIGR4 capsule switch strains
TNF-alpha levels quantitated by ELISA from bronchoalveolar lavage fluid following 4 hours IN inoculation of opaque capsular switch strains. *P* = 0.001 (Kruskal-Wallis) for the overall comparison between strains. *P* values for individual strains compared to the TIGR4(-)+4 strain are given above the respective box and whisker plot (Dunn's multiple comparison test).

5.3 SUMMARY

Flow cytometry and immunogold EM demonstrated increased C3b/iC3b deposition on otherwise isogenic strains expressing serotype 23F and 6A capsules compared to strains expressing the ST4 and 7F capsules; however there was no difference in capsule thickness between strains in the same phase. Furthermore, the phase variation of *S. pneumoniae* strains was found to affect complement activity with increased C3b/iC3b deposition on transparent phase variants. Whilst capsule serotype specific IgG levels correlated with C3b/iC3b deposition on the TIGR4(-)+ capsular switched strains in human test serum, C3b/iC3b deposition was also increased on the TIGR4(-)+6A and +23F strains in naive mouse and baby rabbit serum which contain no *S. pneumoniae* specific IgG. Human serum depleted of C1q (and therefore lacking antibody-mediated complement activity) also showed a similar pattern, suggesting the effects are largely independent of antibody. Furthermore test human serum depleted of IgG function by IdeS also showed a similar pattern of C3b/iC3b deposition on the capsular switch strains. C3b/iC3b deposition results in sera deficient in alternative pathway activity and data on the binding of classical pathway mediators to the TIGR4(-)+ strains, as well as the immunogold EM demonstrating large clusters of C3b/iC3b only on the +6A and +23F strains, all indicated that the differences in C3b/iC3b deposition between strains are mainly alternative pathway dependent (Brown et al. 2002), as has previously been suggested for non-isogenic strains of different capsular serotypes (Winkelstein et al. 1976).

Neutrophil phagocytosis of *S. pneumoniae* is largely complement dependent and there are significant differences in susceptibility to phagocytosis for different *S. pneumoniae* strains (Winkelstein et al. 1976; Kim et al. 1999; Yuste et al. 2008; Melin et al. 2009). Neutrophil

phagocytosis of the TIGR4(-)+ strains correlated closely with the C3b/iC3b deposition results when bacteria were opsonised in serum lacking anti-pneumococcal antibody. The TIGR4(-)+6A strain which had the highest level of C3b/iC3b deposition was also the most sensitive to neutrophil phagocytosis, with an intermediate result for the TIGR4(-)+23F strain and both the TIGR4(-)+4 and +7F strains being relatively resistant to neutrophil phagocytosis. These differences in phagocytosis were abolished when the TIGR4(-)+ strains were not opsonised with C3b/iC3b. Both the TIGR4(-)+6A and +23F strains exhibited reduced virulence in a mouse septicaemia model, with a nearly four-fold difference in recovered bacterial CFU 24 hours after infection with the TIGR4(-)+6A compared to the TIGR4(-)+4 strains, correlating with C3b/iC3b and neutrophil phagocytosis results. Furthermore, the capsular serotype of these strains was found to have an effect in a lung infection model, with the TIGR4(-)+6A and +23F exhibiting decreased survival, increased association with alveolar macrophages and a lower TNFα response within the first 4 hours of lung infection in comparison to the serotype 7F and 4 expressing strains.

CHAPTER 6

THE EFFECT OF NON CAPSULAR GENETIC FACTORS ON COMPLEMENT MEDIATED IMMUNITY

6.1 INTRODUCTION

There is considerable genetic variation between *S. pneumoniae* strains independent of genes required for capsule biosynthesis, due to gene insertions, gene deletions and allelic variation. Molecular epidemiology studies using multi locus sequence typing (MLST) show that non-capsular genetic variation is partially linked to capsular serotype (http://www.mlst.net/), and hence any relationship between capsular serotype and complement resistance may not necessarily be caused by variations in capsule structure. At present the relative importance of capsular serotype or other genetic differences between *S. pneumoniae* strains for complement activity and whether there is a relationship between relative complement resistance and *S. pneumoniae* invasiveness is not known and needs clarification.

To investigate whether non-capsular genetic variation between *S. pneumoniae* strains causes variation in susceptibility to complement, complement deposition on a range of *S. pneumoniae* clinical isolates expressing common capsular serotypes was assessed. Results of these experiments were correlated with the invasive potential of the different strains to assess if there is a relationship between the invasiveness of a strain and its ability to resist complement mediated immunity.

6.2 RESULTS

6.2.1 Measurement of capsule thickness in clinical isolates of *S. pneumoniae*

Clinical isolates were given as a kind gift from Dr William Hanage and Dr Brigitta Henriques-Normark. The strains were all isolated from children under 5 years old with invasive pneumococcal disease and represent a range of serotypes which are relatively highly invasive (1, 4, 14) and serotypes which are relatively lowly invasive (6A, 6B, 9V, 19F and 23F). Serotype 1 is of concern due to its high incidence in empyema in children and the CPS of serotypes 6B and 6A differ in structure by a single bond.

All clinical isolates were grown on Tryptone Soya agar with catalase and examined under a microscope to determine phase of the *S. pneumoniae* strains. All clinical isolates were found to be in opaque phase (data not shown). The Stains-All assay was used to measure the relative amount of capsule polysaccharide produced by the 6B or 23F serotype clinical isolates. There was no significant difference in the amount of polysaccharide quantified between the different MLST strains from within the 6B serotype, nor between strains from the 23F serotype ($P > 0.05$, Fig. 6.1). Furthermore, there was no difference in capsule thickness between selected 6B and 23F clinical isolate strains when viewed by electron microscopy ($P > 0.05$, Fig. 6.2). These results suggest that there is no major difference in capsule expression (ie capsule thickness) between the different strains of these capsular serotypes.

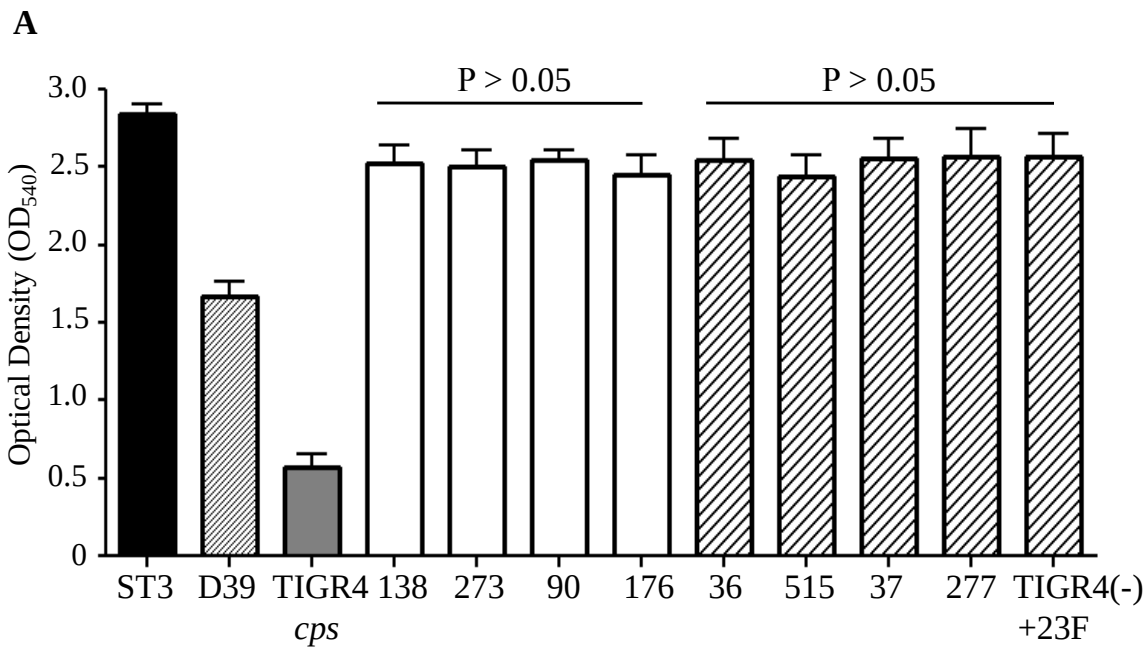
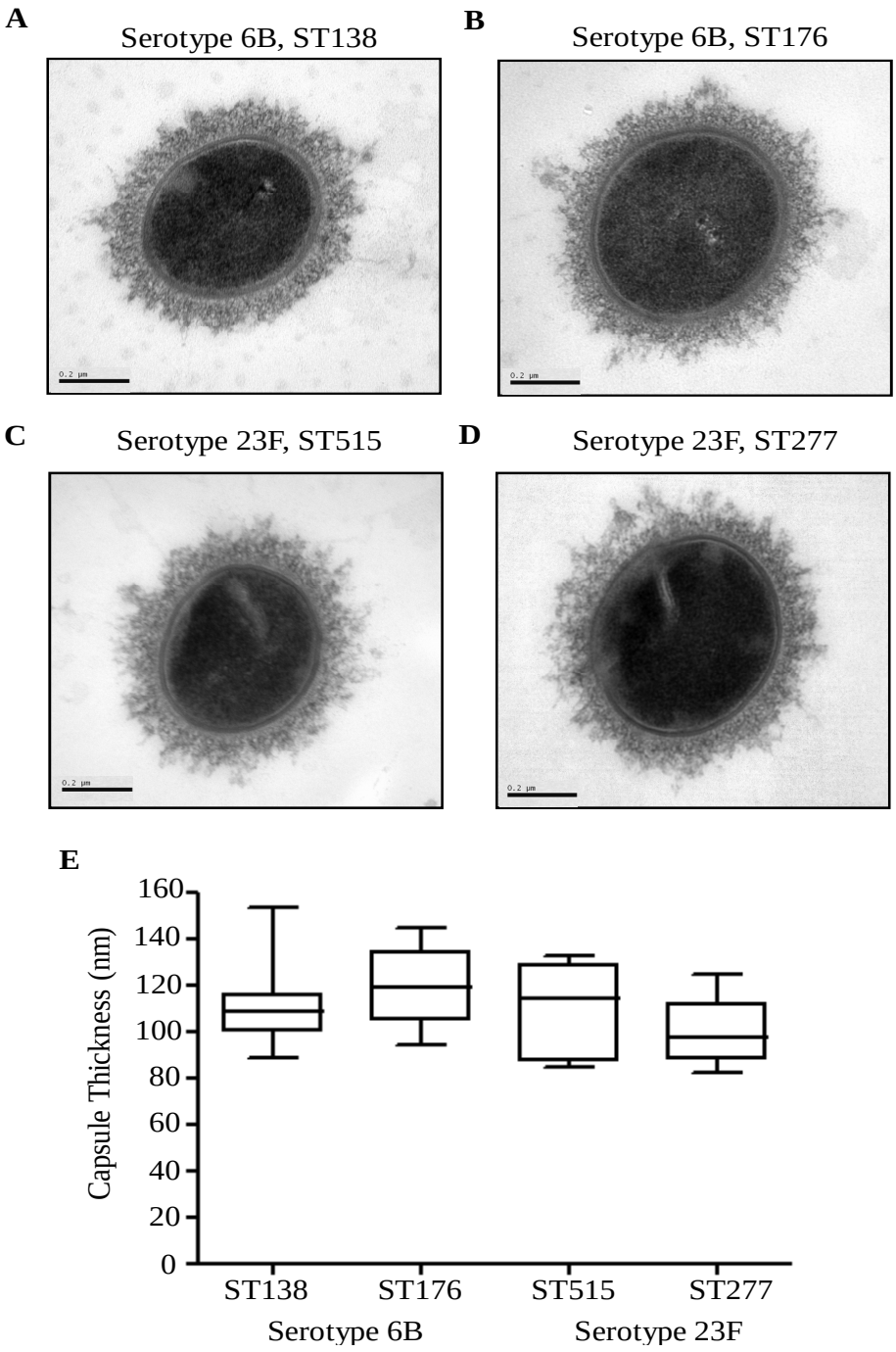


Fig 6.1 Biochemical capsule assessment in serotype 6B and 23F *S. pneumoniae* strains
Stains-All Assay semi-quantitating the amount of bacterium-associated capsule polysaccharide in strains relevant to this thesis chapter. Serotype 3 wild-type, D39 encapsulated TIGR4*cps* strains (black bars) were used as controls and compared to clinical isolates strains of either serotype 6B (open bars) or 23F (slashed bars). There is no statistically significant difference within strains of the 6B or 23F serotype.



<u>Fig 6.2</u> EM capsule measurement in serotype 6B and 23F strains

(A) to (D) Representative EM images of the serotype 6B ST 138 (A), ST 176 (B), and the serotype 23F ST 515 (C) and ST 277 (D) strains respectively. (E) Analysis of capsule thickness of 10 random bacteria per *S. pneumoniae* strain as determined by measuring the total area within the cell wall, the area including capsule polysaccharide and calculating an average capsule thickness using the formula area = Πr^2. Kruskal-Wallis ANOVA shows no statistically significant difference between the strains.

6.2.2 Non-capsular factors influence C3b/iC3b deposition on *S. pneumoniae*

To investigate whether other genetic variation between *S. pneumoniae* strains could affect complement activity independent of capsular serotype, C3b/iC3b deposition was assessed on opaque variants of genetically distinct clinical isolates for the capsular serotypes 4, 6B, 14 and 23F. C3b/iC3b deposition varied between strains within serotypes, most noticeably for 6B and 23F isolates (Fig. 6.3). The serotype 6B ST176 and ST90 strains showed significantly less C3b/iC3b deposition than the ST273 and ST138 strains (ANOVA with post hoc tests $P<0.01$), despite these strains expressing the same capsule polysaccharide with the same capsule thickness. All of the MLST strains from serotype 23F showed a statistically significant difference in C3b/iC3b deposition (ANOVA with post hoc tests $P<0.01$). 4 different MLST isolates from serotype 6A were also assessed for C3b/iC3b deposition, and showed variation with genetic background. However, as with the TIGR4 capsular switch strains (Fig 5.4), there was a high level of C3b/iC3b deposition on the serotype 6A strains in comparison to strains from other serotypes (Fig 6.4). Immunogold EM for C3b/iC3b deposition on selected 6B and 23F strains gave similar results as flow cytometry assays (Fig. 6.5). The serotype 6B isolate of ST138 and the serotype 23F isolate of ST217 with high levels of C3b/iC3b deposition on flow cytometry had 16 (IQR 11-34) and 17 (IQR 12-33) gold particles associated with each bacterium respectively, whereas the 6B isolate of ST176 and 23F isolate of ST515 strains with lower levels of C3b/iC3b deposition on flow cytometry had 5 (IQR 3-9) and 7 (IQR 4-13) gold particles associated with each bacterium respectively ($P=0.01$ for 23F strains, and <0.0001 for 6B strains, Mann Whitney U-test) (Fig. 6.4 E). These data show that there were large variations in C3b/iC3b deposition on *S. pneumoniae* strains independent of capsular serotype.

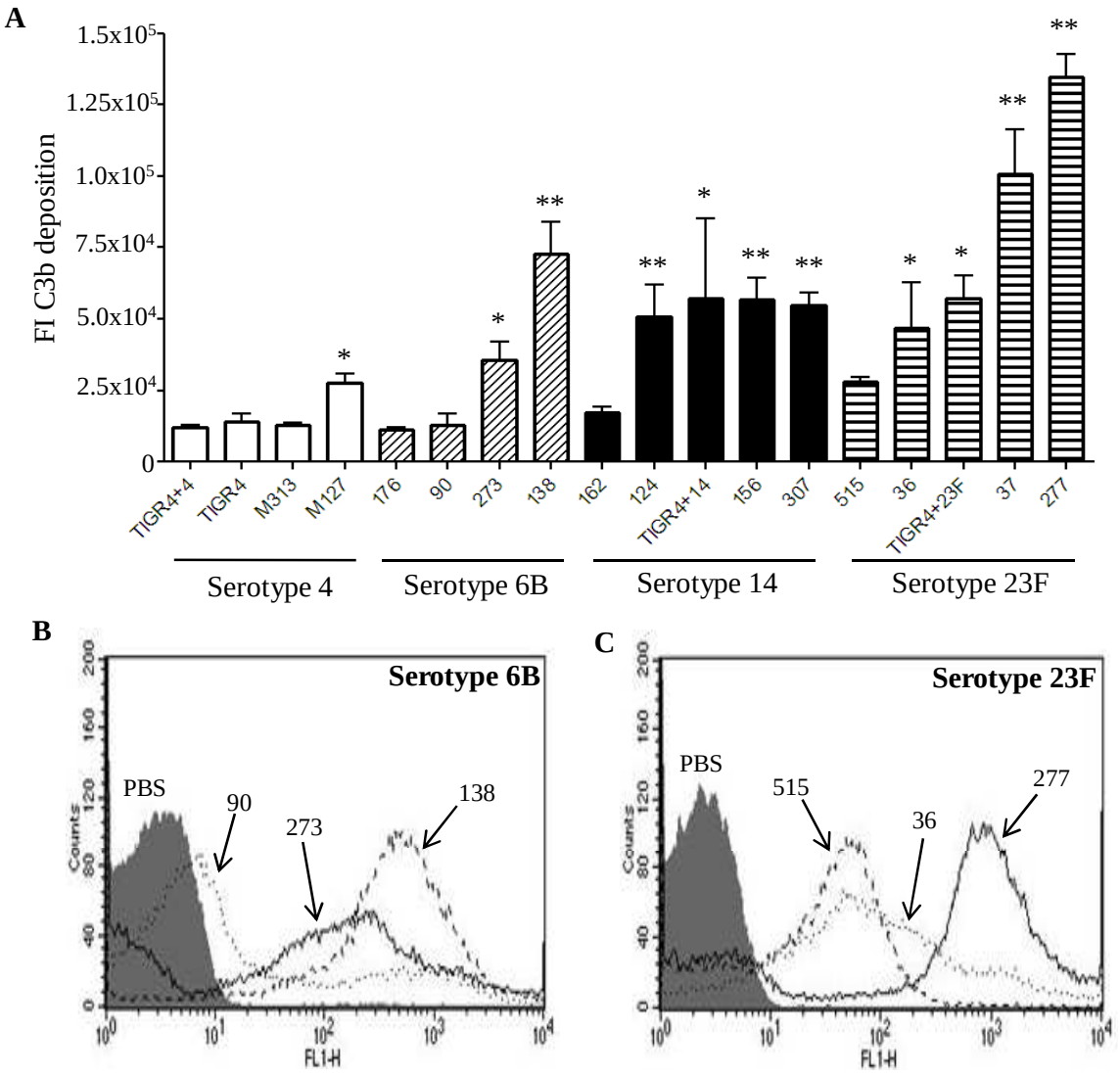
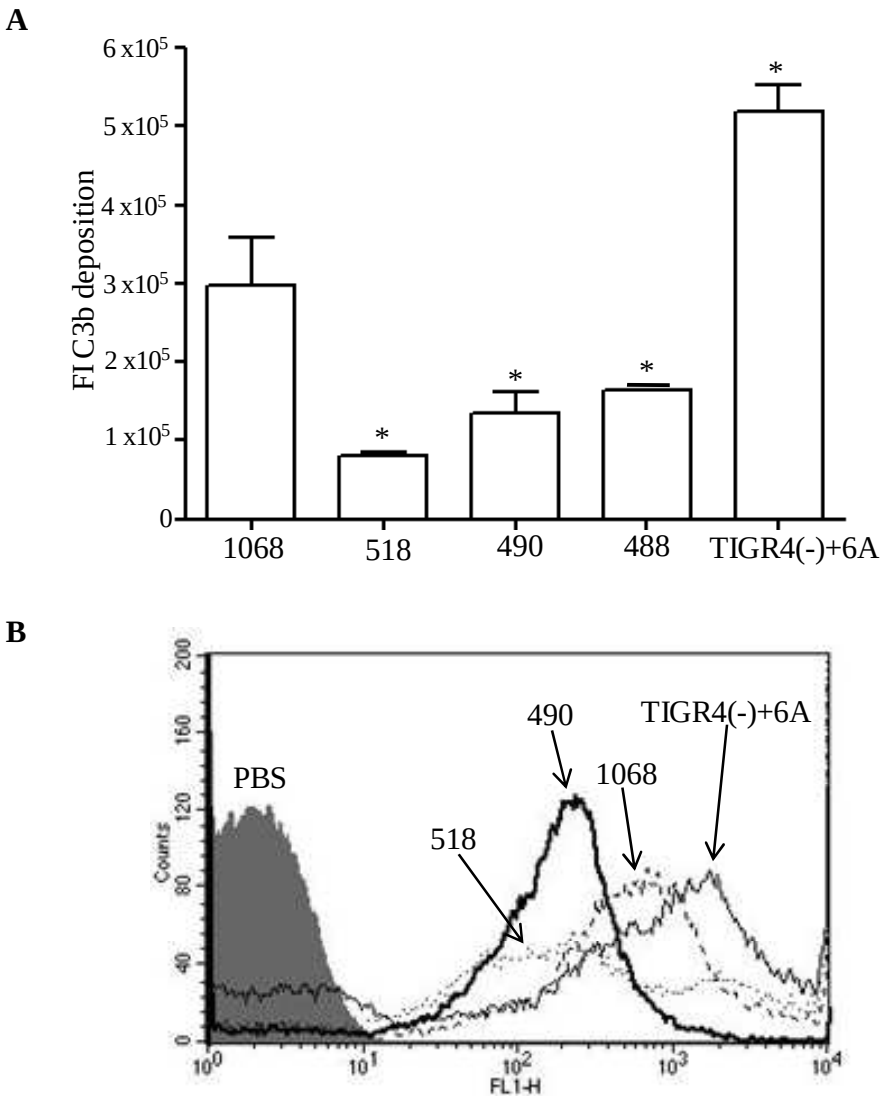


Fig 6.3 Effect of non-capsular serotype genetic variation on C3b/iC3b deposition

(A) Mean (SD) FI of C3b/iC3b deposition measured using flow cytometry on capsular serotype 4 (white bars), 6B (diagonal slash bars), 14 (black bars) and 23F (horizontal slash bars) clinical isolates (labelled with their ST number) in 25% human serum. TIGR4(-)+4 and +23F strains are included for comparison. *P < 0.01, **P < 0.001 (ANOVAs with post-hoc tests) comparing results within a serotype to the TIGR4 (serotype 4), ST176 (serotype 6B), ST162 (serotype 14) and ST515 (serotype 23F) strains. (B), (C) Examples of a flow cytometry histograms for C3b/iC3b deposition on selected serotype 6B (B) and 23F (C) clinical isolates labeled by ST number.



<u>Fig 6.4</u> C3b/iC3b on serotype 6A clinical isolates of *S. pneumoniae*

(A) Mean (SD) FI of C3b/iC3b deposition measured using flow cytometry on capsular serotype 6A clinical isolates (labelled with their ST number) in 25% human serum. TIGR4(-)+6A is included for comparison. * $P < 0.001$ (ANOVAs with post-hoc tests) comparing results to the ST1068 strain. (B) Examples of flow cytometry histograms for C3b/iC3b deposition on serotype 6A clinical isolates labelled by ST number. The histogram for the ST488 strain is not included for ease of interpretation of this figure and because this strain had no significant differences to the ST490 strain.

6.2.3 The effect of capsular serotype on C3b/iC3b deposition on clinical isolates

To further investigate complement interactions with *S. pneumoniae*, C3b/iC3b deposition on 32 *S. pneumoniae* strains representative of both relatively highly (1, 4 and 14) and weakly invasive (6A, 6B, 9V, 19F and 23F) serotypes were compared. The total strains analysed included the clinical isolates described above of serotypes 4, 6B, 14 and 23F plus clinical isolates of capsular serotypes 1, 6A, 9V and 19F and an additional two serotype 6B and one serotype 14 strain (Table 6.1). There was considerable variation in results between strains, and between most serotypes there were marked overlap in results for individual strains. However, C3b/iC3b deposition on the serotype 6A strains remained higher than on other serotypes (median FIs of 134,800 versus 50950 respectively, $P = 0.0054$) (Fig 6.5). There were also differences in C3b/iC3b deposition for isolates belonging to the same clonal complex and with the same capsular serotype that were isolated from different geographical backgrounds (eg serotype 6B isolates of ST138 and ST176), a result that is compatible with recent data showing genetic variation between strains with the same ST and capsular serotype (Silva et al. 2006).

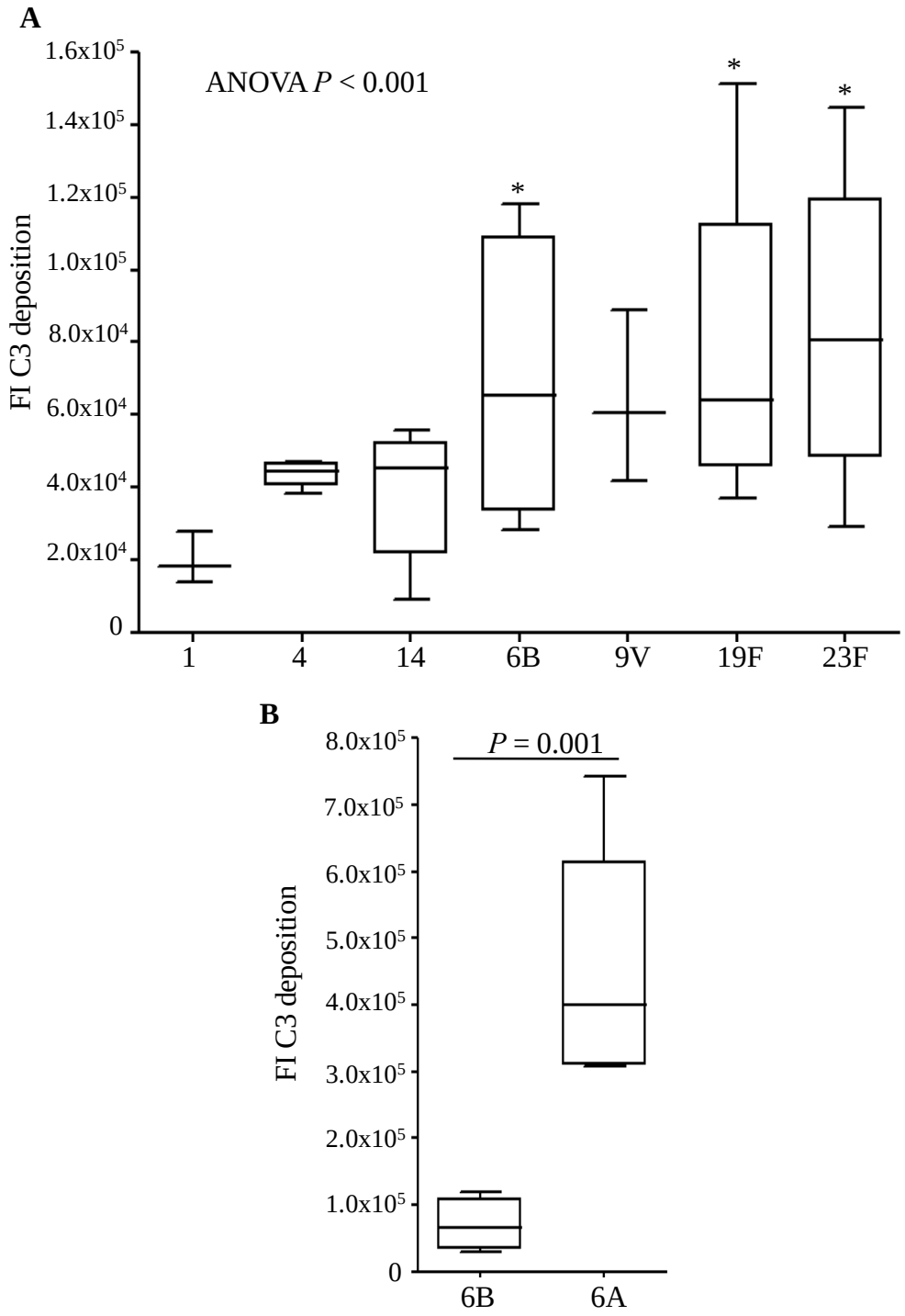


Fig 6.5 C3b/iC3b deposition on different serotypes of *S. pneumoniae*
(A) Median FI of C3b/iC3b deposition measured using flow cytometry for clinical isolate strains from serotypes 1, 4, 14, 9V, 6B, 19F and 23F encompassing between 3-5 different MLST strains per serotype. *P < 0.05 (Kruskal Wallis with Dunn's multiple comparison test) compared to serotype 1. (B) Median FI of C3b/iC3b deposition measured using flow cytometry on 6B and 6A clinical isolate strains, representing the median value for C3b/iC3b deposition obtained from between 3 and 5 strains within each serotype. Mann-Whitney U-test P = 0.001.

Table 6.1 Binding of C3b/iC3b, IgG, C1q and FH to clinical isolates of *S. pneumoniae* and percentage association of the clinical isolate strains with neutrophils when opsonised in 20% human serum.

Serotype	ST	IgG Binding (FI ± SD)[a b]	C1q Binding (FI ± SD)[a b]	FH Binding (FI ± SD)[a b]	C3b/iC3b Binding (FI ± SD)[a b]	% neutrophil association (± SD)
14	124	1520 ± 180	1960 ± 110	1370 ± 160	45224 ± 4790	52.4 ± 4.2
14	162	1660 ± 180	790 ± 140	2650 ± 220	9060 ± 1490	45.0 ± 0.2
14	156	2700 ± 220	2720 ± 300	1480 ± 20	55800 ± 8190	59.2 ± 0.3
14	307	2800 ± 260	4300 ± 170	2410 ± 110	34510 ± 6850	75.4 ± 1.4
14	124	1550 ± 30	4280 ± 140	2960 ± 290	48640 ± 3840	71.1 ± 5.4
23F	36	1450 ± 130	3300 ± 550	1280 ± 120	67080 ± 8280	55.2 ± 4.1
23F	37	2810 ± 220	4030 ± 240	1600 ± 120	94130 ± 9770	71.9 ± 1.9
23F	277	3450 ± 170	6350 ± 430	1020 ± 120	144780 ± 8240	92.3 ± 5.3
23F	515	650 ± 30	910 ± 60	2190 ± 130	29250 ± 1190	45.0 ± 0.5
6A	488	4470 ± 560	6700 ± 390	960 ± 280	484860±10920	65.0 ± 0.9
6A	490	1800 ± 340	3740 ± 330	520 ± 20	313230 ± 9730	75.7 ± 2.2
6A	518	2000 ± 460	4690 ± 390	980 ± 70	307290 ± 8370	47.1 ± 4.2
6A	1068	6940 ± 80	7480 ± 170	480 ± 180	743240±13850	94.6 ± 0.6
6B	90	2920 ± 740	2590 ± 40	3100 ± 30	38460 ± 8910	66.7 ± 4.2
6B	138	1700 ± 330	5990 ± 430	1940 ± 180	83550 ± 10240	65.0 ± 5.0
6B	176	2100 ± 20	3690 ± 330	2100 ± 250	46780 ± 6980	62.0 ± 3.2
6B	273	1160 ± 300	1720 ± 60	1990 ± 70	28330 ± 3500	57.4 ± 0.9
6B	138	4000 ± 200	6780 ± 160	1750 ± 230	118330 ± 1820	95.9 ± 1.9
6B	176	1870 ± 90	5480 ± 70	1160 ± 440	99540 ± 6760	88.3 ± 2.4
1	306	1150 ± 250	4360 ± 130	2908 ± 180	27600 ± 5930	63.9 ± 4.5
1	228	1060 ± 50	1470 ± 120	3870 ± 400	14000 ± 3260	59.8 ± 3.1
1	217	1090 ± 40	2790 ± 265	3760 ± 50	18340 ± 4000	46.2 ± 2.8
4	1222	1360 ± 30	1530 ± 370	2170 ± 100	45590 ± 1780	55.5 ± 3.1
4	205	1650 ± 50	3850 ± 520	2360 ± 160	38170 ± 1720	48.7 ± 3.2
4	205	1440 ± 80	3380 ± 625	1430 ± 100	47130 ± 9080	68.7 ± 4.1
4	259	1390 ± 40	1820 ± 490	1390 ± 160	43060 ± 13090	56.4 ± 3.9
9V	162	1040 ± 100	4340 ± 130	2160 ± 200	41809 ± 8320	76.3 ± 3.0
9V	162	1250 ± 290	4710 ± 880	2560 ± 100	60744 ± 7590	78.3 ± 2.5
9V	156	1260 ± 90	3380 ± 100	1470 ± 250	88814 ± 10140	66.9 ± 4.2
19F	425	800 ± 10	4480 ± 690	2100 ± 150	54470 ± 3950	63.7 ± 2.2
19F	162	1170 ± 120	4220 ± 980	1490 ± 250	36980 ± 4000	63.3 ± 1.9
19F	236	1810 ± 130	4220 ± 600	2430 ± 140	73880 ± 11990	80.3 ± 2.8
19F	556	2610 ± 260	6350 ± 700	1030 ± 110	151280 ± 800	93.5 ± 5.6

[a] FIs are expressed as arbitrary units
[b] IgG, C1q and FH binding was measured in 25% human serum

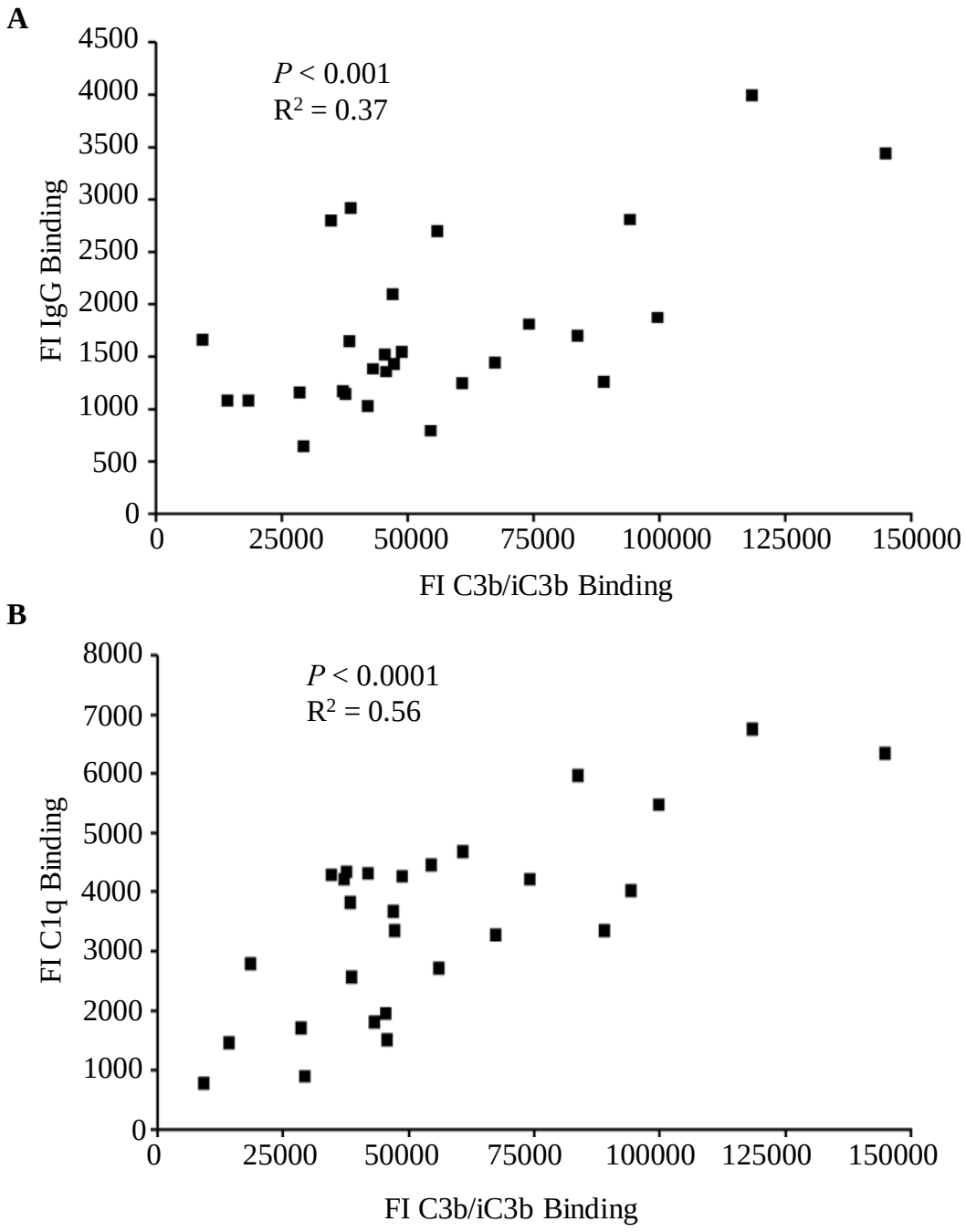
6.2.4 The effect of antibody and complement mediators on C3b/iC3b deposition

The variation in C3b/iC3b deposition between strains within a serotype might be accounted for by differences in the ability of strains to resist interactions with IgG, as well as classical complement pathway mediators (such as C1q) and alternative pathway regulators (FH). Hence the binding of IgG, C1q and FH to the clinical isolates of *S. pneumoniae* was investigated using flow cytometry assays previously described, enabling correlations to be made between binding of these complement regulators and C3b/iC3b binding (Table 6.1). There was variation in the total IgG binding between strains of the same serotype, which may be due to variation in different sub-capsular antigens and hence different levels of specific IgG in the serum.

IgG activates the complement system through the classical pathway by allowing C1q binding to the Fc portion of the antibody molecule and thereby initiating the complement cascade resulting in increased cleavage of C3 into C3b. Total IgG binding to *S. pneumoniae* strains was found to moderately correlate to the results of the C3b/iC3b deposition assays (Pearson's Correlation Co-efficient R^2=0.37) (Fig 6.6 A), although anti-capsular IgG did not correlate to C3b/iC3b (Pearson's Correlation Co-efficient R^2=0.17, *P*=0.34) (data not shown). The IgG was removed from human test serum by pre-incubation with IdeS, which acts to cleave the Fc fragment of the IgG, and used to examine the effect of antibody on C3b/iC3b deposition on the 6B and 23F clinical isolates (Table 6.2). As with previous results, there was decreased C3b/iC3b deposition on all *S. pneumoniae* strains when antibody was removed from the serum, suggesting the importance of antibody in complement mediated immunity against clinical isolates of pneumococcus. The serotype

6B ST176 showed less C3b/iC3b deposition than both the ST138 and ST176 strains in both the control and IdeS treated serum (ANOVAs with post-hoc tests $P < 0.01$), and the serotype 23F ST515 strain showed significantly less C3b/iC3b deposition compared to the ST37 and ST277 strains (ANOVAs with post-hoc tests $P < 0.01$). Hence in both the 6B and 23F clinical isolates the overall pattern of C3b/iC3b deposition persisted in the absence of antibody (Table 6.2).

Furthermore, C1q binding was found to correlate with C3b/iC3b deposition (Pearson's Correlation Co-efficient R^2=0.56) (Fig 6.6 B), and total IgG binding correlated to C1q binding (Pearson's Correlation Co-efficient R^2=0.41, P<0.0001). The alternative complement pathway is regulated by the inhibitory FH, which has been shown to bind CbpA which varies in structure between different *S. pneumoniae* strains. The binding of FH also correlated with C3b/iC3b deposition on these strains of *S. pneumoniae* (Pearson's Correlation Co-efficient R^2=0.37) with increased FH binding associated with decreased C3b/iC3b deposition (Fig 6.7).



<u>Fig 6.6</u> Correlation of IgG and C1q binding to C3b/iC3b deposition

(A), (B) Correlation of FI IgG (A) and C1q (B) to FI C3b/iC3b deposition on clinical isolates of *S. pneumoniae* measured opsonised with 25% human serum and measured using flow cytometry. For both panels, Pearson's Correlation Co-efficient and associated *P*-value are shown on the figure. Original data shown in Table 6.1.

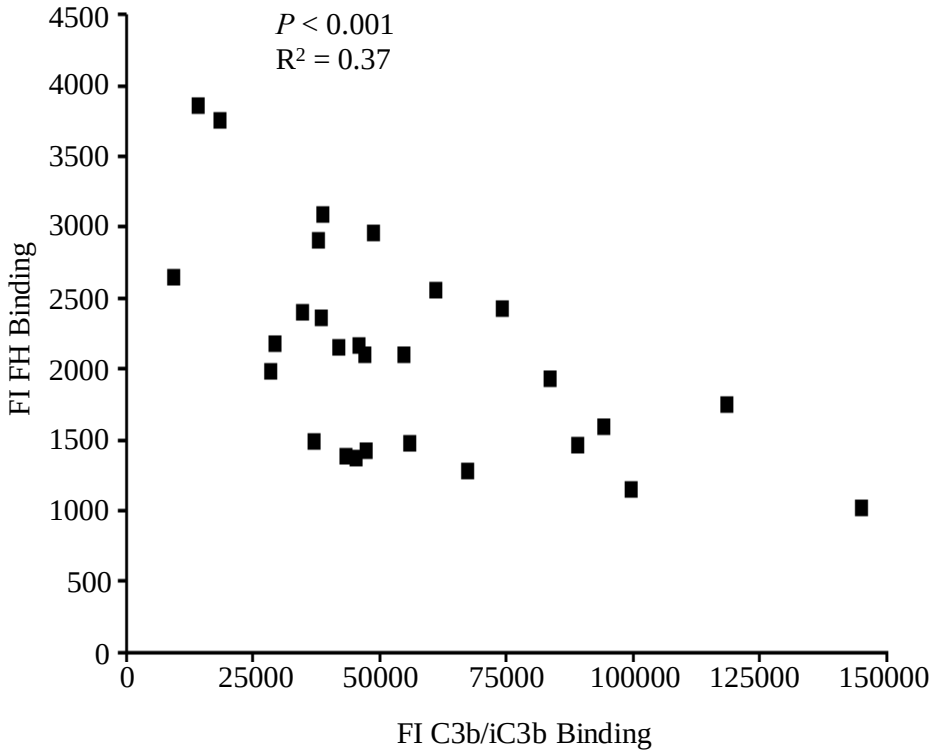


Fig 6.7 Correlation of FH binding to C3b/iC3b deposition
(A) Correlation of FI FH binding to FI C3b/iC3b deposition on clinical isolates of *S. pneumoniae* measured opsonised with 25% human serum and measured using flow cytometry. Original data shown in Table 6.1.

Table 6.2 Effects of IgG depletion using IdeS on mean FI +/- SDs of C3b/iC3b deposition and on *S. pneumoniae* clinical isolate strains from serotypes 6B and 23F in 50% human serum. *P* values represent comparisons between *S. pneumoniae* clinical isolates against the either control (BSA) or IdeS treated ST273 (6B isolates) or ST515 (23F isolates) using ANOVA with Dunnett's multiple comparison test.

Serotype	Strain (ST)	IdeS treated	C3b/iC3b Deposition (FI ± SD)	*P* value
6B	90	No	7420 ± 870	> 0.05
		Yes	3600 ± 680	> 0.05
	138	No	49340 ± 3900	< 0.01
		Yes	25510 ± 4040	< 0.01
	176	No	4960 ± 1100	< 0.01
		Yes	1730 ± 190	< 0.01
	273	No	11220 ± 390	-
		Yes	4240 ± 1190	-
23F	36	No	14790 ± 1390	> 0.05
		Yes	5810 ± 320	> 0.05
	37	No	70470 ± 15840	< 0.01
		Yes	15380 ± 2780	< 0.01
	277	No	87770 ± 5960	< 0.01
		Yes	46010 ± 4310	< 0.01
	515	No	9640 ± 840	-
		Yes	2890 ± 380	-

6.2.5 C3b/iC3b deposition correlates with opsonophagocytosis

To assess whether the differences in C3b/iC3b deposition between *S. pneumoniae* strains had functional consequences, phagocytosis of selected strains was measured using a flow cytometry assay and rabbit complement (to eliminate the effects of varying antibody levels to the different strains). Results were expressed as the proportion of neutrophils associated with fluorescent bacteria, known to be mainly due to phagocytosis (Yuste et al. 2008). The differences between serotype 6B and 23F clinical isolates in the results of the C3b/iC3b deposition assays seemed to persist in the opsonophagocytosis assay (Fig 6.8 A), with strains showing high levels of C3b/iC3b deposition also having high levels of phagocytosis. Furthermore there was a strong relationship between C3b/iC3b deposition in rabbit serum and association of neutrophils with the serotype 23F and 6B strains (Pearson's Correlation Co-efficient R^2=0.952) (Fig 6.8 C). Although there was some variation in the results of the opsonophagocytosis assay between rabbit and human serum, presumably due to the complement-independent effect of anti-pneumococcal antibodies present in human serum, the results correlate significantly (Fig 6.9) (Pearson's Correlation Co-efficient R^2=0.51). To see if this pattern persisted in the presence of antibody, opsonophagocytosis assays were repeated using human serum as an opsonin (Table 6.1). Correlation of C3b/iC3b deposition on *S. pneumoniae* with opsonophagocytosis using human serum found that the relationship between C3b/iC3b deposition and neutrophil association was maintained in human serum, albeit with a weaker correlation than in the absence of antibodies against pneumococcus (Pearson's Correlation Co-efficient R^2=0.58) (Fig 6.9).

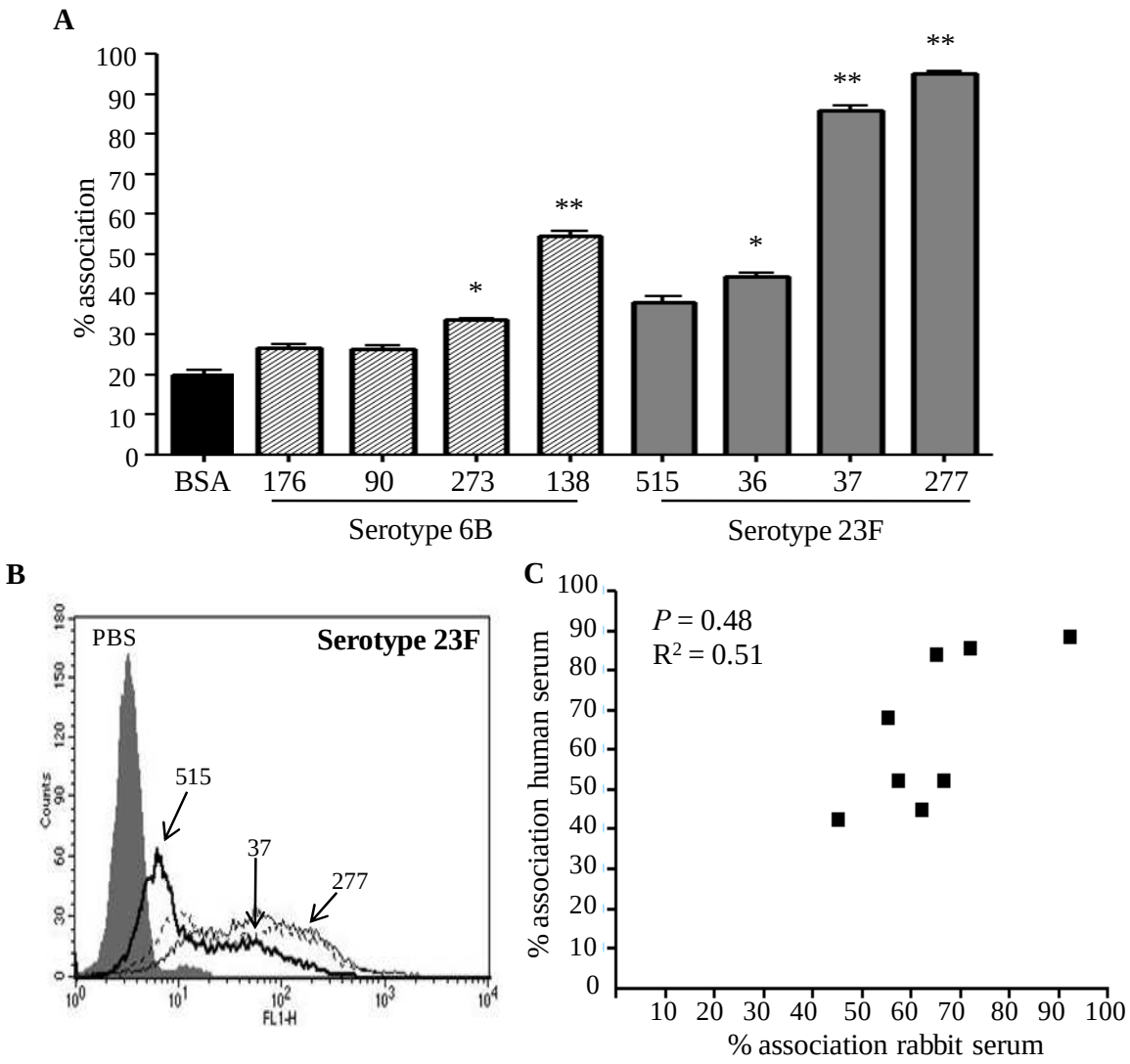
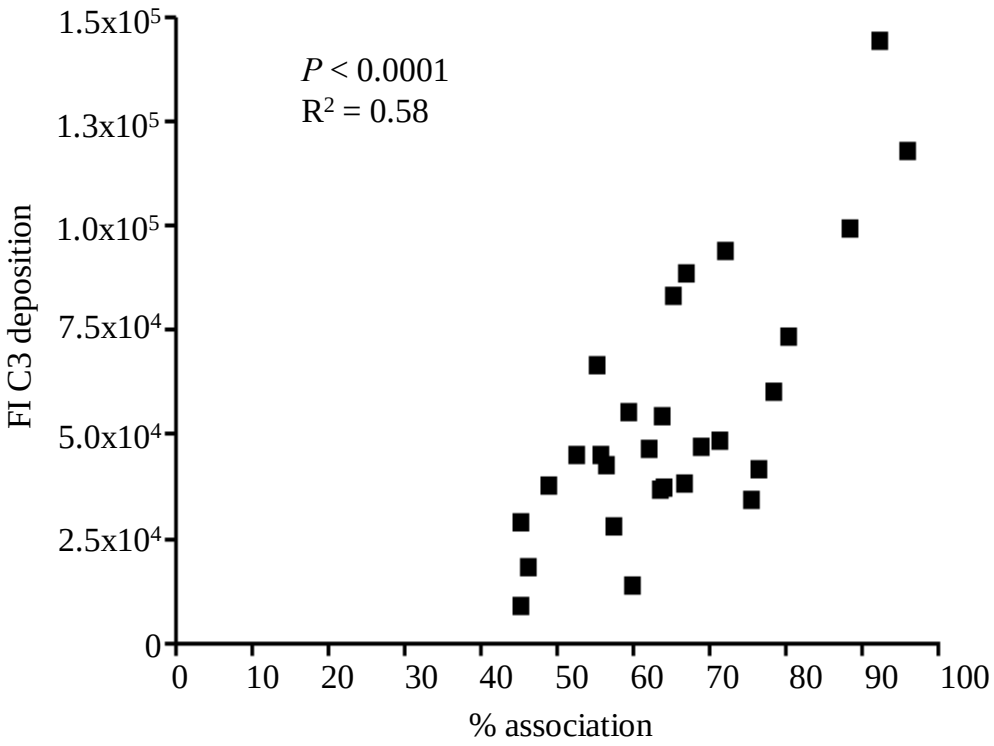


Fig 6.8 Neutrophil phagocytosis of serotype 6B and 23F *S. pneumoniae* clinical isolates
(A) Neutrophil phagocytosis of serotype 6B (slashed bars) and 23F (grey bars) clinical isolates (labelled by ST number) incubated in 20% baby rabbit complement. Results are expressed as the mean (SD) proportion of neutrophils associated with fluorescent bacteria (labelled with FAMSE). Results obtained when bacteria were opsonised in BSA did not significantly differ between strains. **P* < 0.01 or ***P* < 0.001 (ANOVAs with post-hoc tests) compared to ST176 for the serotype 6B strains, and to ST515 for the serotype 23F strains. (B) Examples of flow cytometry histograms for the association of serotype 23F strains with neutrophils after opsonisation with 20% rabbit complement. (C) Correlation between FI of C3b/iC3b deposition measured using flow cytometry and the proportion of neutrophils associated with fluorescent bacteria for the 23F and 6B clinical isolates (ST numbers adjacent to data points) with baby rabbit serum. Error bars represent SDs, and Pearson's Correlation Coefficient was $R^2 = 0.952$ (*P* < 0.001).



<u>Fig 6.9</u> Correlation of C3b/iC3b results and neutrophil phagocytosis in human serum
Correlation between FI of C3b/iC3b deposition measured using flow cytometry and the proportion of neutrophils associated with fluorescent bacteria all clinical isolates using human serum. Pearson's Correlation Coefficient was $R^2 = 0.58$ ($P < 0.001$).

6.2.6 C3b/iC3b deposition correlates with invasiveness

As complement is vital for preventing systemic infection (Yuste et al. 2005) and as capsular serotype affects C3b/iC3b deposition on *S. pneumoniae* I hypothesised that capsular serotypes particularly associated with IPD are likely to be relatively complement resistant. To investigate this possibility, mean C3b/iC3b deposition on 12 *S. pneumoniae* strains representative of relatively highly invasive (1, 4, 7F and 14) and 21 *S. pneumoniae* strains representative of weakly invasive (3, 6A, 6B, 9V, 19F and 23F) serotypes were compared. Overall, median C3b/iC3b deposition on strains from invasive serotypes was significantly lower than the results for strains from weakly invasive serotypes (median FIs of 41600 IQR 17200-55880 versus 88810 IQR 39410-105000 respectively, $P = 0.0075$) (Fig. 6.10 A). The results remained statistically significant even when the serotype 6A strains were excluded from analysis of the weakly invasive serotype group (to prevent the high C3b/iC3b deposition levels on 6A strains skewing data) (median FI 72680 IQR 36400-100200, $P = 0.0036$) (Fig. 6.10 B). Furthermore, the median C3b/iC3b deposition for serotypes which are relatively highly invasive was significantly lower than the median C3b/iC3b for relatively lowly invasive serotypes (median FI 42144 IQR 29152-44775 versus 72885 IQR 62459-29230 respectively, $P = 0.0095$) (Fig 6.11 A). Again these results remained statistically significant when the serotype 6A was excluded from analysis (median FI 65156 IQR 62460-133100, $P = 0.0159$) (Fig 6.11 B). These data show that *S. pneumoniae* capsular serotypes particularly associated with invasive infection are relatively resistant to complement compared to serotypes which are less likely to cause IPD, providing one possible reason why *S. pneumoniae* strains can vary in virulence and providing further evidence for the importance of complement for immunity to *S. pneumoniae*.

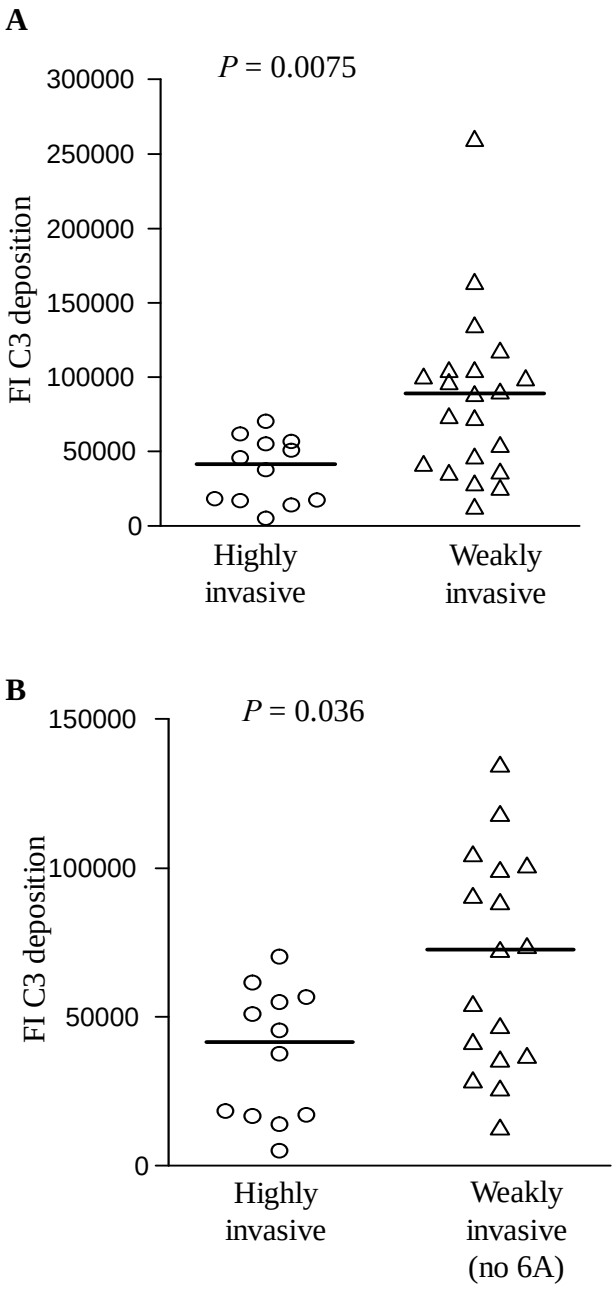
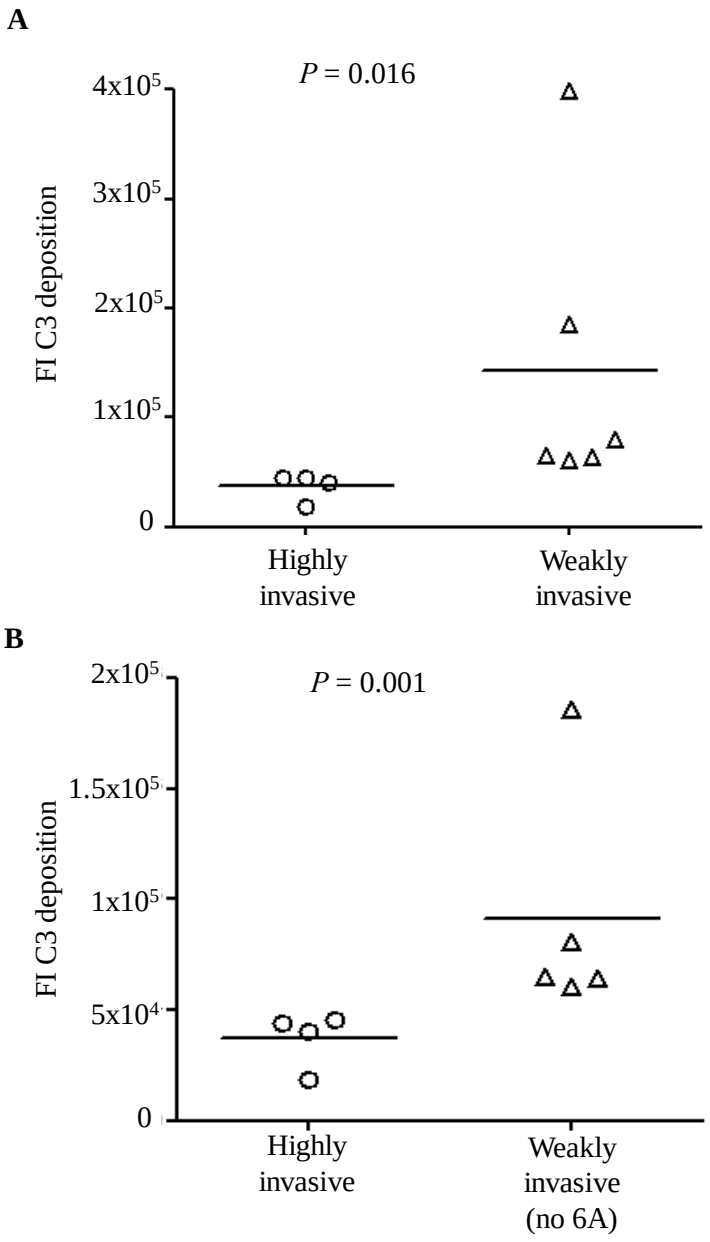


Fig 6.10 Relationship of C3b/iC3b results to invasiveness
(A), (B) FI of C3b/iC3b deposition measured using flow cytometry on all clinical isolates from invasive serotypes (circles, serotypes 1, 4, 7F and 14) compared to the results for all strains from weakly invasive serotypes (triangles, serotypes 3, 6A, 6B, 9V, 19F, and 23F) including (A) or excluding (B) the results for serotype 6A strains. The symbols represent the mean value of three or more assays for each strain. Bars represent the median values for each group. *P* values were obtained using the Mann Whitney U-Test.



<u>Fig 6.11</u> Relationship of C3b/iC3b results to invasiveness by serotype

(A), (B) Median FI of C3b/iC3b deposition measured using flow cytometry from invasive serotypes (circles, serotypes 1, 4, 7F and 14) compared to the median results per serotype for weakly invasive serotypes (triangles, serotypes 3, 6A, 6B, 9V, 19F, and 23F) including (A) or excluding (B) the results for serotype 6A strains. The symbols represent the median value obtained for each serotype. *P* values were obtained using the Mann Whitney U-Test.

6.3 SUMMARY

In summary, using several strains of representative STs for 4 different capsular serotypes the results in this chapter demonstrate that, at least for some capsular serotypes, there were consistent and perhaps surprisingly large variations in C3b/iC3b deposition on *S. pneumoniae* between strains with different genetic backgrounds but the same capsular serotype. The differences in C3b/iC3b deposition were not related to capsule thickness, and correlated with IgG, C1q and inversely correlated with FH binding on *S. pneumoniae*. Furthermore, C3b/iC3b deposition correlated closely with the results of neutrophil phagocytosis assays in both rabbit complement and human serum. There were even differences between strains that although they have the same ST and the same capsular serotype were isolated from different geographical backgrounds (e.g. the ST138 and 176 serotype 6B strains, and the ST124 serotype 14 strains). These data demonstrate that complement activation by *S. pneumoniae* can be markedly affected by non-capsular factors, including differences in recognition of subcapsular antigen by IgG and possibly differing levels of FH binding.

The hypothesis that relative resistance to complement activity may explain some of the differences in virulence between capsular serotypes was investigated by comparing C3b/iC3b deposition on 12 strains from the invasive capsular serotypes 1, 4, 7F and 14 to the results for 21 strains from serotypes with relatively low invasive potential, using strains representative of prevalent STs for each capsular serotype. Strikingly, C3b/iC3b deposition on invasive serotypes was significantly reduced compared to the results for weakly invasive strains. Hence invasive serotypes seem to be less sensitive to complement-dependent bacterial clearance and this could partially explain why these serotypes are particularly

capable of causing systemic infection. These data suggest that the varying effects of different capsular serotypes on complement activity against *S. pneumoniae* are clinically relevant.

CHAPTER 7 DISCUSSION

7.1 DISCUSSION

Although the *Streptococcus pneumoniae* capsule is known to be an essential virulence factor, there is surprisingly little detailed information on how the capsule enables both systemic and lung virulence. There is considerable data which supports the vital role of complement in immunity against *S. pneumoniae*, especially for preventing systemic infection (Gross et al. 1978; Tu et al. 1999; Brown et al. 2002; Jonsson et al. 2005; Yuste et al. 2005; Khandavilli et al. 2008; Yuste et al. 2008), and inhibition of complement activity by the capsule is likely to be important for *S. pneumoniae* virulence. However, little is known about several aspects of the effects of the capsule on *S. pneumoniae* interactions with complement and neutrophils. These include which complement pathways are inhibited, the potential role of known mediators of complement activity, and whether inhibition of phagocytosis by the capsule is just a consequence of inhibition of opsonisation with complement or is also due to the effects of the capsule on other mediators of phagocytosis. Additional phenotypes ascribed to the capsule such as resistance to NETs (Wartha et al. 2007) may also influence virulence, and overall the importance for the development of *S. pneumoniae* invasive disease of inhibition of complement activity by the capsule requires clarification. A more complete understanding of how the capsule can affect virulence would help the investigation of why some capsular serotypes are more able to cause invasive disease in humans (Brueggemann et al. 2003; Brueggemann et al. 2004) and perhaps assist our understanding of the implications of capsular serotype replacement in response to vaccination campaigns (Jacobs et al. 2008).

Both TIGR4 and D39 strains of *S. pneumoniae* have been used extensively for pathogenesis studies and these strains are virulent in mice. The capsular serotype 4 clinical isolate TIGR4 belongs to a relatively invasive serotype and D39 is a laboratory strain of *S. pneumoniae* which was originally derived from an invasive serotype 2 strain and has been one of the main strains used to investigate pneumococcal disease. The unencapsulated TIGR4 strain was constructed by complete replacement of the capsular locus with the Janus cassette (Nelson et al. 2007) whereas the unencapsulated D39 strain was constructed by an in-frame deletion of *cpsD*, a gene that encodes an enzyme required for regulation of capsule synthesis (Morona et al. 2004). Both strains have been previously well characterised, however in this thesis the loss of capsule was confirmed using a biochemical method (Stains-All assay) as well as through visualisation by electron microscopy. Previous studies suggested that deletion of whole or parts of the *cps* locus affect growth of *S. pneumoniae* (Battig et al. 2007) and hence the effect of these mutations on the TIGR4*cps* and D39-DΔ strains was investigated. In contrast to the results from Battig et al, the results showed that loss of capsule had no significant effect on *S. pneumoniae* growth in complete medium (THY broth). This may perhaps be accounted for by the *cpsD* mutation in the D39-DΔ strain being less severe than the mutations analysed by Battig and additionally by the effect of complete disruption to the *cps* locus differing between TIGR4 and D39 strains. Furthermore neither unencapsulated strain exhibited a growth defect in serum, and all strains showed greater growth in serum than in blood at each time point. There was also no apparent growth defect in the unencapsulated strains in blood, which was unexpected given the presence of neutrophils in the blood and the increased opsonophagocytosis of both TIGR4*cps* and D39-DΔ compared to their encapsulated parental strains. However since 2 x 10^6 CFU *S. pneumoniae* were inoculated into 1ml of blood (which contains approximately

6 x 10^3 PMNs/ml) there was a considerable excess of bacteria which overwhelmed the capacity of the neutrophils present in the blood to clear the *S. pneumoniae*.

In accordance with the results of other investigators for the D39 strain (Quin et al. 2007), the flow cytometry analysis of C3b/iC3b deposition on *S. pneumoniae* confirmed that the capsule inhibits C3b/iC3b opsonisation of the D39 strain. Furthermore the TIGR4 capsule was also found to inhibit C3b/iC3b deposition. However the extent to which the capsule prevented C3b/iC3b deposition varied between the D39 and TIGR4 strains, with a larger difference in C3b/iC3b deposition on the TIGR4*cps* strain compared to the TIGR4 than between the D39-DΔ and D39 strains. It is possible that the difference in C3b/iC3b deposition is due to differing levels of antibodies against the different antigens present on the TIGR4 and D39 strains as the level of total anti-pneumococcal antibodies against these strains was not quantitated. However, in the presence of IdeS, which cleaves the Fab portion of IgG and thereby prevents IgG activating complement, there was still a relatively greater increase in C3b/iC3b deposition on the TIGR4*cps* strain compared to the D39-DΔ strain suggesting that this effect is only partially dependent on antibody. Furthermore, since *S. pneumoniae* is not a natural pathogen of mice the results obtained using mouse serum to opsonise the bacteria are independent of antibody effects, providing further evidence that the capsule affects antibody independent complement mechanisms. Whilst this difference may be attributable to the structure of the polysaccharide capsule, the TIGR4 capsule was also thicker than the D39 capsule. There is a large amount of allelic variation between strains in complement inhibitory proteins such as CbpA and PspA (Briles et al. 1981; Crain et al. 1990; Iannelli et al. 2002; Bentley et al. 2006) which could also influence the effects of an individual capsule on complement activity. A full investigation of the relationship of

capsular serotype to complement inhibition would require studying encapsulated and unencapsulated strains from multiple serotypes and perhaps different strains of the same serotype, but would be important given the relationship between capsular serotype and virulence.

The increase in complement activity against unencapsulated strains was confirmed using an immunoblot against C3 to look for C3 breakdown products with different doses of *S. pneumoniae*. For both the TIGR4 and D39 strains there was increased C3 breakdown in the serum at all doses of inoculum used. These results support the flow cytometry data and confirm that the difference in C3b/iC3b deposition detected on the unencapsulated strains is not merely an artefact created by increased antibody access to cell wall surface bound C3b/iC3b in the unencapsulated strains. Furthermore, flow cytometry indicated a decreased ratio of iC3b to total C3b/iC3b on the bacterial surface of both TIGR4 and D39 compared to TIGR4*cps* and D39-DΔ respectively, showing that the capsule inhibits breakdown of C3b to iC3b on the bacterial cell surface.

It is thought that both innate and adaptive immune responses use the classical pathway to initiate C3b/iC3b deposition on *S. pneumoniae* and are activated by recognition of the bacteria by specific IgG, natural IgM and the pentraxins CRP and SAP, whereas the alternative pathway acts mainly to amplify C3b/iC3b deposition (Szalai et al. 1996; Walport 2001; Xu et al. 2001; Brown et al. 2002; Yuste et al. 2007). Flow cytometry data obtained with both human and mouse sera depleted in either the first component of the classical pathway C1q or the alternative pathway protein factor B demonstrated that both

pathways are required for the increase in C3b/iC3b deposition on unencapsulated *S. pneumoniae*.

The capsule prevented recognition of both TIGR4 and D39 *S. pneumoniae* by the classical pathway mediators IgG, IgM and CRP (but interestingly not SAP) and this was associated with an increase in C1q binding. Depletion of IgG using IdeS demonstrated that a substantial portion of the effects of the capsule on inhibition of C3b/iC3b deposition was due to prevention of IgG binding to *S. pneumoniae* and therefore activation of the classical complement pathway. Although, in vaccinated individuals anti-capsular antibody is important for immunity to *S. pneumoniae*, the data showing that IgG binding to *S. pneumoniae* is inhibited by the capsule suggest that most of the naturally occurring IgG in unvaccinated individuals recognises subcapsular antigens, presumably cell wall phosphocholine or cell surface protein antigens (Briles et al. 1981). Hence, as has been demonstrated for specific *S. pneumoniae* antigens and also for other bacteria (McDaniel et al. 1991; Daniels et al. 2006), the capsule masks subcapsular antigens from host IgG and this is one mechanism by which the capsule inhibits classical pathway activation.

The data obtained with human serum depleted of IgG and mouse sera, especially data obtained with *C1qa*$^{-/-}$ sera which cannot support either IgG or natural IgM mediated complement activity (Brown et al. 2002; Yuste et al. 2008), shows persisting differences in C3b/iC3b deposition between encapsulated and unencapsulated strains. Therefore as well as inhibiting antibody-mediated complement activity, the capsule acts to directly inhibit complement activity. The EM immunogold experiments showed large clusters of C3 particles at one site on the cell wall suggestive of focal amplification of complement

activity only in unencapsulated stains. Taken together with the increased CRP, IgG and C1q binding data, these results suggest that the capsule can prevent both recognition of *S. pneumoniae* by the classical pathway mediators IgG and CRP and amplification of C3b/iC3b deposition by the alternative pathway. Prevention of CRP binding to *S. pneumoniae* is a possible IgG-independent mechanism through which the capsule inhibits classical pathway activity, but there are likely to be other mechanisms as well. How the capsule prevents alternative pathway activity is less clear, but mechanical and perhaps charge-charge inhibition of access of complement pathway proteins to *S. pneumoniae* targets beneath the capsule is likely to be important. As expected given that FH binding by *S. pneumoniae* is mediated by the subcapsular proteins PspC and PhtD (Dave et al. 2001; Ogunniyi et al. 2009), FH binding on unencapsulated strains was increased, which would be expected to decrease rather than increase alternative pathway activity. However, the effects of increased FH binding are likely to be outweighed by other capsular effects on complement activity in the alternative pathway. For example the alternative pathway dependent increase in C3b/iC3b deposition on unencapsulated *S. pneumoniae* could reflect amplification of complement activity initiated through increased classical pathway activity, but there may also be direct inhibition of alternative pathway activity by the capsule analogous to other pathogens (Maruvada et al. 2009). The non-IgG mediated mechanisms of capsule inhibition of complement activity require further investigation.

The EM immunogold pictures suggest that complement activation only occurs at a surprisingly limited number of sites on the cell wall of even unencapsulated *S. pneumoniae*. Whether the presence of cell wall complement inhibitory proteins such as PspA and CbpA (Jarva et al. 2002; Yuste et al. 2006) prevent more uniform C3b/iC3b deposition or whether

the C3b/iC3b binding sites are genuinely restricted in number will require further investigation. Variation in the distribution of cell surface proteins could affect the overall pattern of C3b/iC3b deposition. Protein virulence factors have a metabolic cost to the bacterium, and therefore it is likely that they would not necessarily be widely distributed throughout the bacterial surface. Hence sporadic distribution of virulence factors which are a complement target may account for the sporadic C3b/iC3b deposition on *S. pneumoniae*. Localisation of proteins to the bacterial poles is essential for the correct function and regulation of cell division, and it may be that the C3b/iC3b deposition is targeted to a specific protein which is located at in higher concentration at the pole (Lybarger et al. 2001). Heterogeneity within the cell wall of *S. pneumoniae* may prove to be important in the distribution of C3b/iC3b deposited on the bacterial cell surface (Vollmer et al. 2000; Vollmer et al. 2001). Certain pneumococcal enzymes involved in separation of daughter cells during bacterial division have been shown to be more concentrated around the bacterial pole, and these may potentially be a target for C3b/iC3b deposition, which could also explain the tendency for the C3b/iC3b clumps to be localised near the bacterial pole (Vollmer et al. 2001; De Las Rivas et al. 2002).

Neutrophil phagocytosis is considered one of the major elements of immunity to *S. pneumoniae*, and is markedly dependent on *S. pneumoniae* opsonisation with complement (Yuste et al. 2008). Hence the significant increases in phagocytosis of both the TIGR4*cps* and D39-DΔ strains compared to the corresponding encapsulated strains at all concentrations of complement used and in the commercially available C9, C1q and factor B depleted sera was expected due to the effects of the capsule on opsonisation of the bacteria with C3b/iC3b. In normal human serum, there was a greater relative difference in the

neutrophil association with TIGR4*cps* and TIGR4 than that for the D39 and D39-DΔ strains, which paralleled the results of the C3b/iC3b deposition on these strains as measured by flow cytometry. As iC3b is thought to be a more efficient opsonin than C3b, the relative increase in iC3b on unencapsulated *S. pneumoniae* may also contribute to the increase in phagocytosis of these strains (Tohyama et al. 2006). Experiments with cytochalasin D demonstrated that the differences between unencapsulated and encapsulated bacteria were largely due to increased internalisation of unencapsulated bacteria. However as well as complement-dependent effects of the capsule on phagocytosis, there were also significant impairments of the association of encapsulated TIGR4 and D39 with neutrophils when the bacteria were incubated in HBSS, or heat treated or commercially available C3 depleted serum, all conditions in which bacteria are not opsonised with complement. These results show that as well as inhibiting neutrophil phagocytosis by reducing opsonisation of *S. pneumoniae* with C3b/iC3b, the capsule also prevents complement-independent mechanisms of phagocytosis. The increased IgG binding to the unencapsulated strains (despite loss of the target for anti-capsular antibodies) could increase Fc-γ receptor-mediated antibody-dependent phagocytosis, but this would not account for the smaller but consistent and significant differences in phagocytosis seen for bacteria incubated in HBSS alone. Furthermore data obtained with IgG depleted and complement-deficient sera also demonstrated increased phagocytosis of *S. pneumoniae.* These data suggest that the capsule inhibits complement and IgG mediated phagocytosis, but also inhibits bacterial interactions with non-opsonic phagocytic receptors such as mannose or scavenger receptors.

The importance of the effects of the capsule on complement activity during systemic infection was investigated using a mouse model of sepsis and genetically modified mice

which were partially deficient in C3, or C1q (no classical pathway activity) or factor B (no alternative pathway activity). In wild-type mice although both the D39 and TIGR4 strains are highly virulent, even inoculation IP with large numbers of unencapsulated bacteria did not cause disease. In contrast, infection of $C3^{+/-}$ mice with unencapsulated bacteria resulted in significant septicaemia, suggesting that complement activity is required for the effects of the capsule on virulence. This was confirmed using competitive infection experiments, which showed that the unencapsulated strains were avirulent in complement sufficient mice but regained virulence in reduced C3 levels, or the absence of C1q or factor B. However, for both the D39 and TIGR4 strains less than one unencapsulated bacterium was recovered from mouse spleens for every hundred encapsulated bacteria, with an even lower ratio for bacteria recovered from blood. Interestingly in the D39 strain loss of the alternative pathway demonstrated a lower restoration of virulence, and hence this pathway has less of an effect *in vivo*, which is in line with previous data stating that the classical pathway is the dominant pathway for complement mediated immunity against this strain of *S. pneumoniae* (Brown et al. 2002). These data are compatible with neutrophil data showing a significant level of complement-independent phagocytosis, and demonstrate that the effects of the capsule on virulence during sepsis are mediated through both complement and complement-independent mechanisms. Whether the latter is due to capsule inhibition of NETs, phagocytosis mediated by Fc-γ or other neutrophil or macrophage phagocytic surface receptors, or other unknown effects of the capsule requires further investigation.

Unfortunately, throughout this thesis the *in vivo* data were generated using $C3^{+/-}$ mice due to an error which occurred in the back-crossing of the mouse strain, which was only recently identified. This makes interpreting the relative importance of complement

dependent compared to non-complement dependent effects of the capsule difficult. In humans, the $C3^{+/-}$ phenotype is associated with a circulating level of C3 which is approximately 50% that found in $C3^{+/+}$ individuals, and it is likely that this is similar for $C3^{+/-}$ mice. The level of C3 in the serum of $C3^{+/-}$ mice needs to be measured, or experiments repeated using $C3^{-/-}$ mice. Infection with pneumococcus leads to rapid C3 consumption (Biozzi 1961; Brown et al. 1983) and hence the presumed lower level in $C3^{+/-}$ mice could rapidly result in functional depletion of C3.

Pulmonary immunity is distinctly different from systemic immunity and it is likely that AMs play a key role in early immune responses in the lung to *S. pneumoniae* infection. AMs are the resident specialised phagocytic cell in the lung and have previously been shown to mediate both phagocytic and inflammatory responses to *S. pneumoniae* and other pathogens. However the effect of the capsule on early lung responses, in particular those from AMs, remains unclear and this was investigated using a *in vitro* mouse macrophage cell line (RAW 264.7 cells) as well an early pulmonary infection model.

There were significant associations between RAW 264.7 cells and all *S. pneumoniae* strains at 15 minutes and a rapid complement dependent association with RAW 264.7 cells and the unencapsulated strains. There was also a slower complement independent effect of the TIGR4 capsule on association with macrophages since at 60 minutes there was a significant increase in association of unencapsulated *S. pneumoniae* opsonised in complement deficient serum or HBSS compared to encapsulated *S. pneumoniae*. Both encapsulated strains showed a high degree of resistance to association with RAW 264.7 cells, with a maximum of 10% of cells becoming associated with bacteria within 60 minutes regardless

of opsonic conditions. This data suggests that the capsule plays an important role preventing association between *S. pneumoniae* and macrophages and that this effect may involve both complement dependent and independent mechanisms. Macrophages in the lung are able to phagocytose bacteria and opsonisation with complement or antibody is important in this process (Jonsson et al. 1985). Internalised *S. pneumoniae* are killed by AMs and with low inoculae are able to clear *S. pneumoniae* from the lungs without requiring neutrophil recruitment (Gordon et al. 2000; Dockrell et al. 2003).

To determine if the polysaccharide capsule has a role in lung infection a well established pneumonia model was used. For both TIGR4 and D39 the encapsulated parental strains showed increased survival over the unencapsulated strains within the first 4 hours of lung infection. There was a two log reduction in bacterial CFU counts of TIGR4*cps* and a one log reduction of D39-DΔ, continuing the trend that loss of capsule in the TIGR4 strain has a more profound effect than capsule loss in the D39 strain. Flow cytometry analysis revealed a corresponding increase in association of the unencapsulated strains with AMs collected in the BAL fluid, implying that it is increased phagocytosis by AMs which are responsible for the increased clearance of the TIGR4*cps* and D39-DΔ strains. Furthermore there was increased internalisation of TIGR4*cps* by AMs compared to the TIGR4 strain when viewed using a Z-stacking confocal microscopy method. However only a small number of AMs were visualised in this manner and this requires more extensive examination.

In addition there was an increased TNFα response to the TIGR4*cps* and D39-DΔ strains despite a decreased bacterial CFU load at this time point, indicating a much stronger

inflammatory response induced in the absence of the capsule. Both AMS and epithelial cells are capable of producing TNFα and the capsule could potentially affect inflammatory responses by either cell type. Clarification of the effect of the *S. pneumoniae* capsule on AMs in terms of both bacterial clearance and immune response in early lung infection can be investigated using a local AM depletion method via liposomal clodronate (Dockrell et al. 2003). However other investigators have outlined the importance of AMs in the pro-inflammatory response (Franke-Ullmann et al. 1996).

The mechanism by which the capsule could inhibit AM phagocytosis also requires further clarification. Although this could be due to secondary effects on complement, the effect of complement in pulmonary immunity remains unclear since levels of complement in BALF are approximately 10% that found in the circulation (Gross et al. 1978). Furthermore complement factors found in BALF are predominately alternative pathway components and previous data has indicated that the classical pathway is the dominant complement pathway for host immune responses to pneumococcus (Brown et al. 2002). There are also other innate immune mechanisms present in the lung, including opsonisation with surfactant and IgG or cell surface scavenger receptors such as MARCO which could play a role in phagocytosis and hence clearance of *S. pneumoniae* (Dallaire et al. 2001; Arredouani et al. 2004).

To investigate the relative importance of complement for the capsule effects on AMs, early lung infection experiments were repeated in mice with a partial complement deficiency. *C3*$^{+/-}$ mice had increased bacterial CFU of both TIGR4 and TIGR4*cps* within 4 hours of infection, indicating a role for complement in early lung clearance of *S. pneumoniae*.

However there was a greater relative increase in the survival of the TIGR4*cps* compared to the TIGR4 strain which suggests that the capsule prevents an early complement-mediated pulmonary clearance. Decreased bacterial survival of the TIGR4*cps* strain was again accompanied with an increased association with AMs. However there was no statistically significant increase in association of the TIGR4 strain with AMs from wild-type and $C3^{+/-}$ mice. $C3^{-/-}$ mice are more likely to have a more significant phenotype and repeat experiments in $C3^{-/-}$ mice are necessary to fully characterise the role of C3 in the lungs of these mice. In parallel with previous results there was an increase in TNFα levels in the BAL fluid from mice inoculated with TIGR4*cps* compared to TIGR4. Furthermore mice with a partial C3 deficiency showed reduced TNFα response compared to wild-type mice, suggesting a role for complement activation in the inflammatory response. C5a and C3a are potent pro-inflammatory mediators released by complement activation, and also stimulate phagocytosis (Janoff et al. 1999; Walport 2001; Mollnes et al. 2002; Skokowa et al. 2005). Hence increased complement activation could have indirect effects on immunity as well as increased opsonisation of unencapsulated strains. Overall these results suggest that complement may indeed play a role in early lung immune responses and that the TIGR4 capsule may act to prevent complement mediated clearance. However they are difficult to interpret, likely to under estimate the effect of complement and require repetition with $C3^{-/-}$ mice. Furthermore additional work to investigate if these observations are true for *S. pneumoniae* strains other than TIGR4 is required.

The observed difference in the early pulmonary TNFα response to encapsulated TIGR4 and D39 compared to the TIGR4*cps* and D39-DΔ strains respectively was investigated further using RAW 264.7 macrophages. TNFα levels produced by these cells was comparable to

levels noted by other investigators in response to LPS and bacterial stimuli including *E. coli* and *S. pneumoniae* (Yoon et al. 2007; Aldridge et al. 2008; Kang et al. 2009). Time points of 3 and 24 hours were chosen to represent release of pre-formed TNFα and production of TNFα respectively. There was an early serum independent release of TNFα from macrophages stimulated with TIGR4*cps* and D39-DΔ, however at 24 hours both strains induced a mainly complement dependent production of TNFα. However there was a smaller complement dependent effect observable in the D39-DΔ strain at 24 hours than in the TIGR4*cps* strain. Neither the TIGR4 nor D39 strain induced a TNFα level which was greater than 25ng/ml over the 24 hour stimulation, demonstrating the anti-inflammatory properties of encapsulated *S. pneumoniae*.

Since TNFα production is classically mediated through the NFκB pathway (Baldwin 1996), quantitative confocal immunofluorescence assays of NFκB RelA (p65) nuclear translocation were performed on RAW 264.7 cells stimulated with TIGR4, TIGR4*cps*, D39 and D39-DΔ (Noursadeghi et al. 2008). All strains of *S. pneumoniae* induced a translocation of NFκB from the cytoplasm to the nucleus when opsonised in human serum. Furthermore in an MOI dose response, the unencapsulated TIGR4*cps* and D39-DΔ strains induced greater nuclear staining of p65 than the corresponding TIGR4 and D39 strain respectively. Again there was a smaller difference between the D39 and D39-DΔ strain than between the TIGR4 strains. The increased nuclear translocation of NFκB in cells stimulated with TIGR4*cps* and D39-DΔ was found to have a significant complement dependent component, as demonstrated by differences observed between bacteria opsonised with heat-treated (complement deficient) and normal human serum. However, as with previous results, there was a larger complement independent effect in the D39 strains.

NFκB activation is tightly regulated through signals that control the breakdown of IκBα (Baldwin 1996), and immunoblots against IκBα in cell lysates from RAW 264.7 cells stimulated with TIGR4 *S. pneumoniae* opsonised in serum showed a statistically significant reduction in signal when macrophages were stimulated with TIGR4*cps* compared to TIGR4. This confirmed the results of the quantitative confocal immunofluorescence assays and furthermore demonstrated that the TIGR4*cps* stimulation of RAW 264.7 cells showed a similar pattern of IκBα degradation to stimulation with LPS. The IκBα signal did not significantly decrease until after 30 minutes stimulation with the encapsulated TIGR4 strain, indicating a delay in the response of the macrophages to TIGR4 *S. pneumoniae* compared to the unencapsulated strain.

Studies have shown that patients with deficiencies in IRAK4 and NEMO-dependent NFκB activation have a marked increased in incidence of IPD (Ku et al. 2005; Ku et al. 2007; Ku et al. 2007), highlighting the importance of the inflammatory response in immunity to *S. pneumoniae*. Furthermore common polymorphisms in the IκB genes *NFKBIA* and *NFKBIE* genes are associated with increased frequency of *S. pneumoniae* infection (Chapman et al. 2007). However conversely there is also data indicating that mice with increased inflammation have increased susceptibility *S. pneumoniae* (Marriott et al. 2006; Hinojosa et al. 2009). Other investigators have outlined the importance of the MAPK signalling pathways in host immunity to *S. pneumoniae* (Schmeck et al. 2004; N'Guessan et al. 2006; Kang et al. 2009) and I have found that both the p38 and ERK 1/2 pathways are activated after RAW 264.7 macrophages are incubated with TIGR4 *S. pneumoniae*. However, interestingly there were no significant differences in the response to

unencapsulated and encapsulated bacteria for these pathways, contrasting with the results for the IκBα pathway. Hence TIGR4 CPS specifically inhibits the NFκB activation pathway in macrophages. This is a potentially interesting finding which may provide some explanation for the greatly increased susceptibly of individuals with deficiency of IRAK4 and NEMO-dependent NFκB activation to *S. pneumoniae* infections. However, this should be clarified to establish the effect occurs for more than one capsule serotype of *S. pneumoniae*. Furthermore, whether this effect persists in the absence of complement and is dependent on phagocytosis should also be established. Various hypotheses could account for how the *S. pneumoniae* capsule specifically inhibits the NFκB activation pathway in macrophages. It is possible that the reduced NFκB activation is a consequence of complement activation through pro-inflammatory C5a acting on the C5a receptor, and therefore internalisation of *S. pneumoniae* does not necessarily have to occur for the capsule to reduce NFκB activation. However it is also possible that increased bacterial association and/or internalisation of the unencapsulated *S. pneumoniae* specifically activates a certain receptor that signals through the NFκB pathway rather than p38 or ERK 1/2 in response to this stimulus.

It remains unclear which pro-inflammatory macrophage receptors the *S. pneumoniae* capsule affects, but important candidates are TLR (2, 4 and 9) and NOD due to increased phagocytosis. In addition, although CR1 and CR3 mediated phagocytosis is thought to be pro-inflammatory (Klickstein et al. 2000; Xia et al. 2000), phagocytosis mediated by the Fcγ receptor is pro-inflammatory and furthermore increased phagocytosis may increase NOD activation. Increased complement activation could also be pro-inflammatory through increased C3a and C5a mediated inflammation. The data on the *S. pneumoniae* capsule

affecting different aspects of complement and phagocytosis suggest capsule would also affect a range of cell-surface pro-inflammatory receptors. How the capsule may differentially affect inflammation requires more detailed investigation using assays in which phagocytosis in inhibited using cytochalasin D. Furthermore macrophages isolated from MyD88-/-TRIF-/- mice in which there is no TLR mediated cell signalling would provide insights as to whether this receptors are involved in the inflammatory response to *S. pneumoniae,* as well as *in vivo* studies in these mice. Experiments carried out using individual TLR knock-out mice and reporter cells which were previously transfected with TLRs would also indicate the role of these receptors in macrophage activation.

Clinical and epidemiological data suggest that different *S. pneumoniae* strains vary in their ability to cause severe disease and that this is linked to capsular serotype, with nasopharyngeal colonisation with strains expressing some capsular serotypes associated with a greatly increased incidence of septicaemia and meningitis (Brueggemann et al. 2003; Brueggemann et al. 2004; Sandgren et al. 2004; Hanage et al. 2005). However, the recent genome sequence data showing considerable genetic variation between *S. pneumoniae* strains unrelated but linked to capsular serotype (Hakenbeck et al. 2001; Hiller et al. 2007) suggest phenotypic differences between strains cannot be assumed to be due to the effects of the capsular serotype alone. Therefore otherwise isogenic bacterial strains expressing four different capsular serotypes were used to investigate the influence of capsular serotype independent of other genetic variation on *S. pneumoniae* interactions with the host immune response.

The flow cytometry and immunogold EM data shows increased C3b/iC3b deposition on otherwise isogenic strains expressing serotype 23F and 6A capsules compared to strains expressing the serotype 4 and 7F capsules. Capsule thickness and phase variation influence complement-mediated immunity to *S. pneumoniae* (MacLeod et al. 1950; Nelson et al. 2007), but the strains investigated were opaque phase variants and had similar capsule thickness when measured using EM. The results contrast with those from Weinberger et al, who found that TIGR4 capsular switch strains expressing capsular serotypes representing a wide range of *S. pneumoniae* strains did show differences in capsule thickness (Weinberger et al. 2009). Different methodology was used by Weinberger et al to determine capsule thickness than those in this thesis, as they used an approach based upon the Quellung Reaction with FITC-Dextran exclusion. It is intriguing that our results differ, and this highlights the issue of quantitating the amount of polysaccharide capsule expressed by a given strain of *S. pneumoniae*. It is possible that the FITC-Dextran methodology is subject to variation in the ability of different capsule polysaccharides to swell, and due to their chemical structure it is also possible that light is refracted differentially by different capsule polysaccharides thereby creating artificial differences in the capsule thicknesses. The EM method used in this thesis is novel, and part of the fixation method requires the dehydration of the sample preparations. It is possible that different capsular polysaccharides have different levels of hydration, and therefore this step may artificially create no apparent difference between capsule thicknesses. Furthermore it is possible that the strategy of selecting spherical and non-dividing bacteria has in some way biased the data towards a particular thickness of capsule. The results of the Stains-All assay for the opaque strains are towards the maximum end of the measurable optical density range, and hence may not accurately reflect differences between the capsular switch strains. However, both the EM

and the Stains-All assay showed significantly less capsule polysaccharide expressed by the transparent phase variants compared to the respective opaque phase TIGR4(-)+ *S. pneumoniae* strain, which correlates with data published by other researchers (Weiser et al. 1994; Kim et al. 1998). Hence EM can distinguish the capsule thickness between strains in different phenotypes and may provide an accurate method to quantitate the thickness of the capsule polysaccharide. An ELISA method has used by other researchers, though this relies upon capsule polysaccharide extracts to quantitate the CPS, and these preparations are known to be contaminated with teichoic acid and other cell wall components (Kim et al. 1998). It would be helpful to have a standardised methodology for quantitating the thickness or amount of polysaccharide capsule expressed by a strain, but all current methodologies have drawbacks and no-one method seems preferable.

There were differences in the binding of C1q, CRP, SAP and FH to the TIGR4 capsular switch strains. However the binding of these complement mediators did not correlate with the C3b/iC3b results, providing no potential explanation for the increased C3b/iC3b on the TIGR4(-) +6A and +23F strains relative to the TIGR4(-) +7F and +4. The differences in the binding levels of these complement mediators were very small, and it is questionable whether these differences would be immunologically relevant. In keeping with the results of the TIGR4, TIGR4*cps*, D39 and D39-DΔ strains, there was increased C3b/iC3b deposition on the transparent phase variant TIGR4(-)+ strains which express a thinner polysaccharide capsule than the corresponding opaque TIGR4(-)+ strains. Furthermore the results of the complement mediator binding to TIGR4 capsular switch strains also mirrored the results of the unencapsulated TIGR4 and D39 strains. There was increased binding of

C1q, CRP, and FH on the transparent phase strains and reduced SAP binding when compared to the opaque phase variants.

Antibodies to capsular polysaccharide increase complement deposition on *S. pneumoniae*, and indeed capsule serotype specific IgG levels did correlate with C3b/iC3b deposition on the TIGR4(-)+ capsular switched strains in the human test serum. The levels of anti-capsular serotype specific antibody in the test serum did not correlate with the total IgG binding as detected by flow cytometry. It should be noted that the fluorescent indexes are quite low for these results, and it would ideally be best to replicate this data set with a whole cell ELISA to measure total antibody binding to the capsular switch strains (Roche et al. 2007). However if this result is accurate it suggests that antibody against non-capsular antigens significantly contribute to the total IgG deposition on a given strain of *S. pneumoniae*. C3b/iC3b deposition was also increased on the TIGR4(-)+6A and +23F strains in both baby rabbit and mouse serum which contain no *S. pneumoniae* specific IgG and in human serum deficient in C1q and therefore lacking antibody-mediated complement activity, suggesting the effects are largely independent of antibody. Repeated C3b/iC3b flow cytometry assays using human test serum which was depleted of IgG using IdeS confirmed that there was still increased C3b/iC3b deposition on the TIGR4(-)+6A and +23F strains relative to the TIGR4(-)+7F and +4 strains when the effect of antibody was removed from human serum. Overall, these results suggest that the differences between C3b/iC3b deposition on the TIGR4 capsular switch strains cannot be accounted for by differing levels of anti-pneumococcal antibody present in the serum.

Furthermore, C3b/iC3b deposition results in sera deficient in alternative pathway activity and data on the binding of classical pathway mediators to the TIGR4(-)+ strains, as well as the immunogold EM demonstrating large clusters of C3b/iC3b on the +6A and +23F strains only, all indicate that the differences in C3b/iC3b deposition between strains are alternative pathway dependent (Brown et al. 2002). The importance of the alternative pathway has previously been suggested for non-isogenic strains of different capsular serotypes (Winkelstein et al. 1976). Overall this data provide evidence that differences in C3b/iC3b deposition between isogenic strains expressing different capsular serotypes reflect differences between capsular serotypes in inhibiting complement activity, and support existing data showing that expression of capsular serotype 3 in a serotype 2 strain affected complement deposition (Abeyta et al. 2003).

Neutrophil phagocytosis of *S. pneumoniae* is largely complement dependent and there are significant differences in susceptibility to phagocytosis for different *S. pneumoniae* strains (Winkelstein et al. 1976; Kim et al. 1999; Yuste et al. 2008; Melin et al. 2009; Weinberger et al. 2009). Therefore neutrophil phagocytosis of the TIGR4(-)+ capsular switched strains was investigated. When the effect of antibody was removed from human serum using IdeS the TIGR4(-) +6A and +23F strains were more sensitive to neutrophil phagocytosis compared to the TIGR4(-) +7F and +4 strains, although in the presence of antibody this pattern was not apparent. Furthermore, mouse serum or baby rabbit complement was used to opsonise the bacteria to prevent the whole cell and capsular specific antibody levels in human serum confounding the results through antibody-Fcγ receptor interactions. Neutrophil phagocytosis of the TIGR4(-)+ strains correlated closely with the C3b/iC3b deposition results. The TIGR4(-)+6A strain which had the highest level of C3b/iC3b

deposition was also the most sensitive to neutrophil phagocytosis, with an intermediate result for the TIGR4(-)+23F strain and both the TIGR4(-)+4 and +7F strains being relatively resistant to neutrophil phagocytosis. These differences in phagocytosis were abolished when the capsular switch strains were not opsonised with C3b/iC3b, confirming they were due to complement activity rather than other potential differences between capsular serotypes in their interactions with non-complement phagocytic receptors. The results of the phagocytosis data suggest that variations between *S. pneumoniae* strains in their sensitivity to phagocytosis can be partially dependent on capsular serotype and complement-sensitivity.

Intriguingly, the TIGR(-)+ strains expressing serotypes associated with invasive *S. pneumoniae* infection (ST4 and 7F) had the lowest level of C3b/iC3b deposition, suggesting perhaps that resistance to complement-mediated immunity may influence invasiveness. However, whether capsular serotype effects on complement truly correlate with *S. pneumoniae* invasive potential can only be answered using a much larger range of capsular switched strains. The recent Weinberger et al paper found that differences in the structure of the capsular polysaccharides correlated to the prevalence of difference *S. pneumoniae* serotypes, and suggested this was due to the different energy costs of the polysaccharides (Weinberger et al. 2009). In addition these investigators demonstrate that the TIGR4(-)+4 strain is relatively poor at surviving non-opsonic PMN killing, whereas the TIGR4(-)+6A and +7F an intermediate survival and the TIGR4(-)+23F strain shows significant survival (Weinberger et al. 2009). However whether the differences in polysaccharide structure affect complement mediated immunity was not investigated. Precisely how different capsular serotype structures can have varying effects on C3b/iC3b

deposition is not clear; ST6A and 23F capsules may allow greater access of complement to the bacterial surface, but capsular serotype could also influence complement deposition indirectly by modifying the interactions of surface proteins such as PspA and CbpA with complement factors (Tu et al. 1999; Yuste et al. 2005; Quin et al. 2007).

Complement is an essential component of the host immune response to systemic infection with *S. pneumoniae* (Winkelstein 1981; Tu et al. 1999; Brown et al. 2002; Jonsson et al. 2005; Yuste et al. 2005; Yuste et al. 2008), and the differences in C3b/iC3b deposition between the TIGR4(-)+ strains would therefore be predicted to have significant consequences for virulence. Indeed the TIGR4(-)+6A and +23F strains had reduced virulence in a mouse model of sepsis, with a nearly four-fold difference in recovered bacterial CFU 24 hours after infection with the TIGR4(-)+6A compared to the TIGR4(-)+4 strains. These results support previously published data showing that expression of capsular serotype 3 can decrease, increase or have no effect on virulence in a mouse model of sepsis depending on the strain background, and show that capsular serotype can affect virulence independent of other genetic variation between strains (Kelly et al. 1994). However the effects of expression of different capsular serotypes affecting the virulence of the TIGR4(-)+ strains were relatively weak, and all these strains still caused significant infection in mice. This contrasts with previous data showing that clinical isolates of serotype 7F and serotype 23F are not virulent in mice (Briles et al. 1992; Sandgren et al. 2005). Furthermore there are marked differences in virulence in mice between *S. pneumoniae* strains of the same capsular serotype (Briles et al. 1992; Sandgren et al. 2004). These data suggest that although capsular serotype can modulate virulence there is also a considerable, perhaps dominant, effect of non-capsular genetic variation on the

development of *S. pneumoniae* infections in mice. The importance of complement for systemic immunity to *S. pneumoniae* makes it plausible that the increased C3b/iC3b deposition and neutrophil phagocytosis of the TIGR4(-)+6A and +23F strains are responsible for the reduced virulence of these strains. However, the differences between capsular serotypes in C3b/iC3b deposition could also correlate with effects on a wider range of host protein / bacterial interactions, for example recognition by macrophages via the lectin SIGN-R1 (which specifically recognises the *S. pneumoniae* capsule) or other non-complement cell surface receptors, which may also influence virulence in the mouse model (Winkelstein et al. 1976; Winkelstein 1981; Arredouani et al. 2004; Kang et al. 2006).

The intranasal infection model showed that the TIGR4(-) +6A and +23F strains have reduced virulence in the lung compared to the TIGR4(-) +7F and +4 capsular switch strains. There was also increased association with AMs of the TIGR4(-) +6A and +23F capsular strains in comparison to the +7F and +4 expressing strains. These results show a similar pattern to the results of the C3b/iC3b binding assay and the PMN association assays. However, the effect of expression of different capsular serotypes was again relatively weak, indicating that other non-capsular genetic factors have an impact on pulmonary virulence of *S. pneumoniae* in mice. Furthermore there was also an increased TNFα response to the TIGR4(-) +6A and +23F strains despite the reduced bacteria survival. These suggest these capsular serotypes are less anti-inflammatory. Overall the capsular serotype TIGR4(-) +6A and +23F have a phenotype closer to the TIGR4*cps* than the TIGR4(-) +7F and +4 i.e. these capsule serotypes are less effective at preventing host/pathogen interactions. Hence the affects of the *S. pneumoniae* capsule on AM response

is likely to vary with capsular serotype, and may affect the ability of different *S. pneumoniae* serotypes to cause respiratory infections.

Currently there are only limited data on the mechanisms by which the conjugate vaccine may induce protection against *S. pneumoniae* pneumonia, obtained mainly with a serotype 1 strain only (Jakobsen et al. 1999; Saeland et al. 2001; Jakobsen et al. 2002). Innate immune responses have a significant impact on adaptive immune responses to vaccines (Ross et al. 2000; Ferreira et al. 2009; Jones et al. 2009), suggesting that serotype-dependent effects on innate immunity may also affect the efficacy of the vaccine in inducing immunity to *S. pneumoniae*. This leads to the hypothesis that the relative resistance to innate immunity of the capsular serotype 4 and 7F capsular switch strains may lead to a reduced efficacy of conjugate vaccines against these serotypes. At present the relative importance of capsular serotype or other genetic differences between *S. pneumoniae* strains for complement activity and whether there is a relationship between relative complement resistance and *S. pneumoniae* invasiveness is not known and needs clarification. Therefore the effect of non-capsular genetic variation on complement mediated immunity was investigated using clinical isolate strains of *S. pneumoniae* which represented several serotypes with different clones from each capsular serotype.

Clinically isolated strains of *S. pneumoniae* were obtained from previous epidemiology studies of invasive pneumococcal disease in children under 5 years old in Sweden and Finland (Sandgren et al. 2004; Hanage et al. 2005). Strains were inclusive of isolates from serotypes 1, 4, 14, 9V, 6B, 6A, 19F and 23F. Serotypes 1, 4 and 14 represent serotypes

which molecular epidemiology studies show have a high invasive disease potential whereas serotypes 9V, 6B, 6A, 19F and 23F have a relatively low invasive disease potential but a high disease incidence presumably due to increase carriage rates (Brueggemann et al. 2003; Sandgren et al. 2004; Hanage et al. 2005; Sjostrom et al. 2006). Furthermore at least 3 different ST strains with known MLST profiles were available from each of these serotypes, which enabled experiments to be performed to assess the relative contribution of capsular serotype and non-capsular genetic variation to the resistance of *S. pneumoniae* to complement deposition and phagocytosis.

Experiments performed with clinical isolates of *S. pneumoniae* also show that C3b/iC3b deposition varies with capsular serotype. Mean C3b/iC3b deposition varied with capsular serotype, although there was also variation in C3b/iC3b deposition between different clones of the same serotype which was not sufficient to affect a serotype dependent difference. It is difficult to state whether this is due to differences in capsule thickness between isolates of different serotypes. However, all the clinical isolates where in opaque phase variation, suggesting a relatively thick capsule, and EM showed no variation in the degree of encapsulation between two strains from a 6B and 23F serotype background (Kim et al. 1998; Weiser et al. 2001). Furthermore the Stains-All biochemical assay revealed no difference in the amount of capsule associated by either the 6B or 23F strains, although since this assay relies on detection of acidic polysaccharides it is not necessarily an appropriate assay to compare degrees of encapsulation between different serotypes consisting of different monosaccharides (Edstrom 1969; Hammerschmidt et al. 2005). As previously discussed, it is relatively difficult to quantitate the amount of capsule polysaccharide expressed by *S. pneumoniae,* although this should be attempted for all the

clinical isolate strains to determine if these results are truly independent of capsule thickness. However, since C3b/iC3b deposition does vary by serotype in strains of *S. pneumoniae* which have been isolated from invasive pneumococcal disease, this suggests that differences in the ability of a given serotype to resist complement deposition may have a functional consequence in disease states. The differences in C3b/iC3b deposition on the *S. pneumoniae* strains correlated closely with the results of neutrophil phagocytosis assays, demonstrating a functional significance for the ability to resist complement deposition.

C3b/iC3b deposition also varied on *S. pneumoniae* strains which had the same capsular serotype but a different genetic background. This was demonstrated using several strains of representative STs for 4 different capsular serotypes. There were consistent and perhaps surprisingly large variations in C3b/iC3b deposition on *S. pneumoniae* between strains with different genetic backgrounds but the same capsular serotype. There were even differences between strains that although they have the same ST and the same capsular serotype were isolated from different geographical backgrounds (eg the ST138 and 176 serotype 6B strains, and the ST124 serotype 14 strains). These results concur with data obtained by a previous student in the lab (Sophia Opel) who performed similar assays in 2004. This gives a consistency to the data despite the different investigators, date of experiments and stock sets used suggesting that this result is likely to be accurate.

Antibody levels against the different *S. pneumoniae* serotypes varied, as did total IgG binding to the different strains. There was an overall positive correlation between IgG binding and C3b/iC3b deposition, suggesting the importance of the antibody effect for

differences in C3b/iC3b deposition between strains. However this correlation of antibody to C3b/iC3b is only moderate, and after the removal of IgG from serum the differences in C3b/iC3b deposition between strains was maintained, indicating that the strain variation in resistance to complement deposition is at least partially independent of IgG.

Interestingly there were differences in the level of IgG binding between strains of the same serotype but different ST, indicating the importance of non-capsular antigens in eliciting and modulating the antibody response to *S. pneumoniae*. These results indicate that non-serotype dependent differences between strains can have quite marked effects on complement activity. Recent data using micro-arrays has shown that strains with the same ST and serotype are not necessarily genetically identical (Dagerhamn et al. 2008). This is a potentially powerful finding, as the genetic variation between strains with the same serotype and ST is relatively limited and therefore the genetic basis for differences in C3b/iC3b deposition could be more readily identified. There are several potential mechanisms for non-capsular serotype variation in C3b/iC3b deposition, including the known allelic variation in *S. pneumoniae* proteins that affect complement activity such as PspA and CbpA, the effects of as yet unidentified proteins encoded by regions of diversity present only in selected strains, and varying expression between strains of target molecules for thioester bond formation with C3. Furthermore, the significant difference in C3b/iC3b deposition between 6A and 6B strains whose capsular loci only differ by a single polymorphism in *wciP* encoding a rhamnosyl transferase (Mavroidi et al. 2004) perhaps suggests that subtle differences in capsule structure that do not affect capsular serotype could still influence complement activity. Identifying which of these potential mechanisms influence non-capsular serotype-dependent variation in C3b/iC3b deposition will be

complex, but may help clarify why strains with otherwise similar genetic backgrounds vary in their virulence potential, analogous to recent data on variations in sensitivity to complement for *Neisseria meningitides* strains (Uria et al. 2008).

Hence I also investigated binding of the complement factors C1q and FH between difference isolates of *S. pneumoniae*. Binding of both the classical pathway mediator C1q and the alternative pathway mediator FH also varied between different STs with the same capsular serotype. C1q binding correlated with C3b/iC3b deposition, and this correlation was in fact tighter than that between IgG binding and C3b/iC3b deposition. This suggests that direct C1q binding to direct targets such as pneumolysin and phosphorylcholine also significantly affects the total C3 binding to a strain along with binding to IgG (Paton et al. 1984; Mitchell et al. 1991; Agrawal et al. 2001).

FH was found to inversely correlate with C3b/iC3b deposition on the *S. pneumoniae* clinical isolates. FH is an inhibitor of the alternative pathway and binds mainly to CbpA, and the considerable allelic variation of CbpA could lead to variation in its ability to bind FH (Iannelli et al. 2002; Quin et al. 2006; Lu et al. 2008). This result conflicts with previous data from other investigators, and this may be due to the reduced numbers of strains that Melin et al investigated compared to the strain library used in these experiments, or simply the result of analysis of different *S. pneumoniae* strains (Melin et al. 2009). If the result in this thesis is correct, it suggests that activity of CbpA is likely to be important in mediating complement resistance in pneumococcal disease. To investigate this further would require sequencing CbpA from the clinical isolates to try and correlate differences in the derived amino acid sequence with variation in FH binding. In addition

mutant strains of the clinical isolates lacking the CbpA protein could be used to measure the consequence of loss of this virulence factor from clinically isolated strains.

Given the vital role for complement in preventing systemic infection by *S. pneumoniae* (Brown et al. 2002; Yuste et al. 2005) and the wide variation in complement activation between strains it was hypothesised that relative resistance to complement activity may explain some of the differences in virulence between capsular serotypes. To investigate this hypothesis C3b/iC3b deposition was compared on 12 *S. pneumoniae* strains from the invasive capsular serotypes 1, 4, 7F and 14 to the results for 21 strains from serotypes with relatively low invasive potential, using strains representative of prevalent STs for each capsular serotype. C3b/iC3b deposition on invasive serotypes was significantly reduced compared to the results for weakly invasive strains. Hence invasive serotypes are on average less sensitive to complement-dependent bacterial clearance and this could partially explain why these serotypes are particularly capable of causing systemic infection. There was a wide range for results of C3b/iC3b deposition on strains from weakly-invasive serotypes which significantly overlapped with the results for invasive serotypes, consistent with the observation that weakly-invasive serotypes are genetically more diverse than invasive serotypes (Sandgren et al. 2004). These data suggest that the varying effects of different capsular serotypes on complement activity against *S. pneumoniae* are clinically relevant. Although complement resistance is likely to be only one of many factors influencing differences in invasive potential between *S. pneumoniae* strains, serotype-dependent effects on complement-dependent immunity and invasiveness are of particular importance as existing vaccines are serotype specific and cause profound changes in the ecology of different *S. pneumoniae* capsular serotypes.

7.2 SUMMARY

In summary the results presented in this thesis show:

1. The *S. pneumoniae* capsule can affect several aspects of complement activity against *S. pneumoniae*, including inhibition of recognition of the bacteria by the classical pathway mediators IgG and CRP, and reducing degradation of C3b bound to the bacterial surface to iC3b.

2. The effects of the capsule on C3b/iC3b deposition results in reduced phagocytosis of encapsulated bacteria, but the capsule also inhibits non-complement dependent mechanisms of neutrophil phagocytosis. These data clarify the contribution of the capsule to *S. pneumoniae* immune evasion.

I have also shown:

3. The capsule is vital for evasion of evasion of early pulmonary immune responses, and affects complement dependent and independent interactions with alveolar macrophages.

4. The capsule affects the inflammatory response of alveolar macrophages, which may contribute to its effect on virulence. Interestingly, although *S. pneumoniae* stimulate the NFκB, p38 and ERK1/2 innate activation pathways the capsule seems to specifically modulate activation of the NFκB transcription pathway. This finding requires further investigation to clarify the mechanisms involved.

5. Opsonisation with C3b/iC3b of *S. pneumoniae* TIGR4(-)+strains varied markedly with capsular serotype, with a higher level on capsular serotypes associated with weakly invasive strains. Differences between capsular serotypes in opsonisation with C3b/iC3b

were correlated closely with differences in neutrophil phagocytosis and in virulence in mice. Overall, this thesis indicates that capsular serotype can affect *S. pneumoniae* resistance to host immune responses, and suggest this maybe one possible cause of some of the variation in invasive potential between strains of this important pathogen. Interestingly, experiments using otherwise isogenic bacteria expressing different capsular serotypes demonstrated that serotypes associated with low frequency of invasive disease showed more rapid pulmonary clearance and induced lower inflammatory responses than serotypes frequently associated with invasive disease.

6. Data from the clinical isolated *S. pneumoniae* strains indicate that opsonisation of with C3b/iC3b varies markedly with capsular serotype and with strain variation independent of capsular serotype, and is associated with the invasive potential of different capsular serotypes. These results suggest that differing sensitivity to host immunity can explain some of the differences in invasiveness between *S. pneumoniae* strains, and may represent a step forward in our understanding of why particular *S. pneumoniae* strains frequently cause invasive disease. In addition, the importance of complement for immunity to a variety of bacteria suggests these results have broader implications for understanding variations in virulence between strains of other bacterial pathogens as well as *S. pneumoniae*.

CHAPTER 8 REFERENCES

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APPENDIX 1

C_{den} MEDIUM

200ml	C_{den} Base	
50ml	HYR	
10ml	1mg/ml Glutamine	(Sigma)
10ml	Vitamins without choline solution	
5ml	2% Sodium Pyruvate	(Sigma)
40ml	SAC Solution	
13ml	Supplement	
15ml	1M KPO_4, pH 8.0	(Sigma)
10ml	10mg/ml Leucine	(Sigma)
5ml	10mg/ml Phenylalanine	(Sigma)
9ml	10mg/ml Lysine	(Sigma)
2ml	1mg/ml Choline	(Sigma)

The above reagents were filtered through 0.45µm filter to produce sterile medium.

SUPPLEMENT

60ml	'3 in 1' Salts	
120ml	20% Glucose	(Sigma)
6ml	50% Sucrose	(Sigma)
120ml	2mg/ml Adenosine	(Sigma)
120ml	2mg/ml Uridine	(Sigma)

The above reagents were filtered through 0.45µm filter to produce a sterile solution.

SAC

12g	NaCl	(Sigma)
12g	Anhydrous sodium acetate	(Sigma)
	Distilled water to a final volume of 1L	

VITAMINS WITHOUT CHOLINE

12ml	Adam's I solution	
32ml	5mg/ml Asparagine	(Sigma)
36ml	Distilled water	

The above reagents were filtered through 0.45µm filter to produce a sterile solution.

HYR

640mg	Histidine	(Sigma)
122mg	Tyrosine	(Sigma)
800mg	Arginine	(Sigma)
	Distilled water to a final volume of 1L	

The above reagents were filtered through 0.45µm filter to produce a sterile solution.

C_{den} BASE

190mg	Glycine	(Sigma)
350mg	Alanine	(Sigma)
720mg	Valine	(Sigma)
760mg	Isoleucine	(Sigma)
1160mg	Proline	(Sigma)
590mg	Serine	(Sigma)
450mg	Threonine	(Sigma)
310mg	Methionine	(Sigma)
140mg	Tryptophan	(Sigma)
720mg	Asppartic acid	(Sigma)
2200mg	Glutamic acid	(Sigma)
150mg	L-Cysteine	(Sigma)
	Distilled water to a final volume of 2L	

The above reagents adjusted to pH 7.0 and were filtered through 0.45µm filter to produce a sterile solution.

ADAM'S I SOLUTION

60µl	0.5mg/ml Biotin	(Sigma)
30mg	Nicotinic Acid	(Sigma)
35mg	Pyridoxine	(Sigma)
120mg	Calcium Pantothenate	(Sigma)
32mg	Thiamine HCl	(Sigma)
14mg	Riboflavin	(Sigma)
	Distilled water to a final volume of 200ml	

The above mixture was filtered through 0.45µm filter to produce a sterile solution, which was stored in the dark.

'3 IN 1' SALTS

100g	$MgCl_2 \cdot 6H_2O$	(Sigma)
0.5g	Anhydrous $CaCl_2$	(Sigma)
200µl	0.1M MnSO4	(Sigma)
	Distilled water to a final volume of 1L	

The above mixture was autoclaved to ensure sterility.

PUBLICATIONS ARISING FROM THIS THESIS

Full Papers:

1. 'Impaired opsonisation with C3b and phagocytosis of *Streptococcus pneumoniae* in serum from subjects with defects in the classical complement pathway.', J. Yuste, A. Sen, L. Truedsson, G. Jönsson, L. S. Tay, **C. Hyams**, H. E. Baxendale, M. Botto and J. S. Brown. Infection and Immunity. 9th June 2008.

2. 'Inhibition of complement-mediated immunity to *Streptococcus pneumoniae* by PspC is dependent on strain background.', J. Yuste, S. Khandavilli, N. Ansari, K. Muttardi, L. Ismail, **C. Hyams**, J. N. Weiser , T. Mitchell and J. S. Brown. Infection and Immunity. In Press.

3. 'The *Streptococcus pneumoniae* capsule has multiple effects on complement activity and bacterial interactions with neutrophils.', **C. Hyams**, E. Camberlein, J. M. Cohen, K. Bax and J. S. Brown. Infection and Immunity. In Press.

4. '*Streptococcus pneumoniae* resistance to complement-mediated immunity is partially dependent on capsular serotype.', **C. Hyams**, K. Bax, J. Yuste, E. Camberlein, J. N. Weiser and J. S. Brown. Infection and Immunity. In Press.

5. '*Streptococcus pneumoniae* resistance to complement is dependent on capsular serotype and and is associated with invasiveness.', **C. Hyams**, S. Opel, J. Yuste, W. P. Hanage, B. G. Spratt, B. Henriques-Normark, J. S. Brown (manuscript in preparation).

Abstracts:

1. ***Hyams C****, Yuste J, Weiser JN and Brown JS*. Effects of the capsule on the interactions of *Streptococcus pneumoniae* with complement. Federation of Infection Societies Conference, Nov 2007.

2. ***Hyams C****, Yuste J, Noursadeghi M, Michael C, Weiser JN and Brown JS*. The capsule prevents both classical and alternative pathway activity against *Streptococcus pneumoniae* and is vital for evasion of early pulmonary immune responses. International Symposium on Pneumococci and Pneumococcal Disease-6, June 2008

3. ***Hyams C****, Opel S, Hanage WP, Yuste J, Spratt B, Weiser JN and Brown JS*. Effects of the capsule on interactions of *Streptococcus pneumoniae* with complement. International Symposium on Pneumococci and Pneumococcal Disease-6, June 2008

4. ***Hyams C****, Opel S, Yuste J, Hanage WP, Henriques-Normark B, Weiser JN, Spratt BG, Brown JS*. Invasive capsular serotypes of *Streptococcus pneumoniae* are relatively resistant to complement compared with non-invasive serotypes. British Thoracic Society Dec 2008

5. ***Hyams C****, Opel S, Yuste J, Hanage WP, Henriques-Normark B, Weiser JN, Spratt BG, Brown JS*. Invasive capsular serotypes of *Streptococcus pneumoniae* are relatively resistant to complement compared with non-invasive serotypes. British Thoracic Society Dec 2008

6. ***Hyams C****, Camberlein EM, Yuste J, Noursadeghi M, Michael C, Wieser JN, Spratt BG, Brown JS*. The *Streptococcus pneumoniae* capsule is essential for evasion of early alveolar-macrophage pulmonary mediated immunity. British Thoracic Society Dec 2008

7. ***Hyams C****, Camberlein EM, Yuste J, Bax K, Weiser JN, Brown JS.* The capsule prevents both alternative and classical pathway activity against *Streptococcus pneumoniae* and is vital for systemic infection. EuroPneumo 2009

8. ***Hyams C****, Bax K, Yuste J, Camberlein EM, Weiser JN, Brown JS.* Invasive capsular serotypes of *Streptococcus pneumoniae* are relatively resistant to complement compared to non-invasive serotypes. EuroPneumo 2009

9. ***Hyams C****, Camberlein E, Noursadeghi M, Weiser JN, Brown JS*. The *Streptococcus pneumoniae* capsule inhibits macrophage activation through the NFκB and not MAPK activation pathways. British Thoracic Society Dec 2009

10. *Camberlein E,* ***Hyams C****, Weiser JN, Khandavilli S, Cohen JM, Brown JS*. The *Streptococcus pneumoniae* capsule protects alveolar macrophage mediated early lung innate immunity. British Thoracic Society Dec 2009

ACADEMIC AWARDS ARISING FROM THIS THESIS

1. The British Association of Lung Researchers Young Scientist Award
BALR Summer Conference 2008.

2. The British Thoracic Society Medical Student Abstract Prize
BTS Winter Conference 2008.

3. University College London Graduate School
Student Conference Travel Award 2008.

4. The Cordwainer's Prize for MB PhD Thesis
UCL 2009.